Effects of Metformin and Combinatory Derived Factors of Adipose Tissue on Prostate Cancer Growth

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Abstract

Obesity is a growing concern worldwide and is associated with increased risk of prostate cancer (PCa) and poor patient prognosis. Metformin (MET) is reported to be protective against PCa in type 2 diabetics as they are at a reduced risk of developing PCa and have a better prognosis if they do in fact develop PCa. My thesis examined the effects of MET on two prostate cancer cell lines, LNCaP and PC3 cells. MET caused alterations in morphology and cell detachment in both cell lines. MET counteracted the effects of leptin which was independent of AMPK activation. PC3 cells incubation with conditioned media and treated with MET caused increased AMPK activation and decreased AKT activation. MET was found to act independently of AMPK although it caused an increase in AMPK activation in PC3 cells. It appears that MET represents a promising adjuvant therapy for obese PCa patients.

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I leave you all now with great and fond memories as I venture onto the next portion of my career, as I go to St. George's University School of Medicine.

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BMI = BODY MASS INDEX
WHR = WAIST TO HIP RATIO
WHO = WORLD HEALTH ORGANIZATION
BAT = BROWN ADIPOSE TISSUE
WAT = WHITE ADIPOSE TISSUE
LEP = LEPTIN
ADIPO = ADIPONECTIN
JAK/STAT = JANUS KINASE/SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION
DIO = DIETARY-INDUCED OBESITY
CNS = CENTRAL NERVOUS SYSTEM
CSF = CEREBROSPINAL FLUID
LMW = LOW MOLECULAR WEIGHT
MMW = MEDIUM MOLECULAR WEIGHT
HMW = HIGH MOLECULAR WEIGHT
gADIPO = GLOBULAR ADIPONECTIN
AMPK = AMP-ACTIVATED PROTEIN KINASE
PPARA = PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR
PI3K = PHOPHOINOSITIDE-3-KINASE
T2D = TYPE 2 DIABETES
RP = RESTRICTION POINT
CDK = CYCLIN-DEPENDENT KINASE
RB = RETINOBLASTOMA TUMOR SUPPRESSOR
UB = UBIQUITIN
LKB1 = LIVER KINASE B1
PCA = PROSTATE CANCER
DRE = DIGITAL RECTAL EXAMINATION
PSA = PROSTATE SPECIFIC ANTIGEN
ADT = ANDROGEN DEPRIVATION THERAPY
LHRH = LUTEINIZING HORMONE-RELEASING HORMONE
CT = COMPUTERIZED TOMOGRAPHY
MET = METFORMIN
CC = COMPOUND C
CM = CONDITIONED MEDIA
ACM = CONDITIONED MEDIA GENERATED FROM ABDOMINAL VISCERAL ADIPOSE TISSUE
PCM = CONDITIONED MEDIA GENERATED FROM PERIPROSTATIC/PERIRENAL ADIPOSE TISSUE

1.0 Introduction:

Obesity is a growing global concern. There are several measures of obesity such as body mass index (BMI), waist to hip ratio (WHR) and body fat percentage with the most commonly used criteria due to its ease of measurement and calculation being BMI (weight in kilograms divided by the square of the height in metres squared (kg/m^2)). A person is classified as overweight if their BMI is 25 to 29.9, and obese if they have a BMI of 30 or greater as defined by the World Health Organization (WHO).

Obesity is most prevalent in western countries such as the United States, where it is reported that 35.7% of people above the age of 20 are obese, with women having a slightly higher rate than men at 36.3% compared to 35.5%[1]. Canada is slightly better off than United States in terms of obesity, where nearly a quarter (23%)[2] of all adults are obese. However, obesity is not only seen in North America. Obesity is a major concern in countries like Australia where more than 60% of adults are obese[3]. Although obesity in China is only a third of the prevalence of that found in Australia, it has increased by over 400% in the past 20 years in China compared with only a 20% increase in Australia over the same period[3]. Obesity has increased at a staggering rate in areas where obesity was previously not normally seen.

In 2008 it was reported that 1.46 billion adults worldwide were determined to be overweight, with 502 million classified as obese[4]. The effects of this obesity pandemic are far reaching as it burdens societies with premature mortality, morbidity associated with many chronic disorders and negative effects on health-related quality of life[5]. This places a financial strain on the system in multiple ways; whether it is paying for the direct cost of medical treatment associate with obesity and the myriad of comorbidities associated with it, or by having

to pay disability for people who are too obese to work or the amount of lost productivity that can be attributed to obesity. In in 1997 it was estimated that the direct cost of obesity in Canada was over $1.8 billion, corresponding to 2.4% of the total health care expenditures[6], with an additional $2.7 billion in estimated indirect costs. An updated study found that the direct cost attributed to being overweight and obesity was $6.0 billion, which is 4.1% of total healthcare expenditure, with $3.9 billion attributed solely to obesity[7]. Also the total indirect cost associated with obesity and its comorbidities to be a staggering $52.6 billion for just 2006[7]. Canada is not isolated in this trend of increasing cost of obesity. A systemic review found that obesity accounted for 0.7-2.8% of the total health-care costs and that obese individuals had medical costs 30% higher than those with normal weight[8]. The increased prevalence of obesity is estimated to account for 27% of growth in US healthcare expenditure from 1987 to 2001[9], and it is projected to account for 16-18% of total US healthcare expenditure by 2030[10].

Aside from the medical cost, society faces the burden of indirect costs of obesity by employees missing work (absenteeism) or reduced productivity. In Sweden, obese individuals were 1.5-1.9 times more likely to take sick leave than were individuals with a healthy weight[11]. In the US alone the loss of productivity due to obesity is estimated to be as high as $390-580 billion in loss to the economy[12].

The etiological causes for the obesity pandemic are still being investigated. However, it is believed that two factors play a major role which are 1) a global shift towards a “Western diet”, high in simple carbohydrates, energy-dense and saturated fats (animal fats), and 2) a rapid decrease in the demand of physical activity in daily life according to the WHO. Regardless of the cause of obesity it is associated with numerous severe health risks including hypertension, coronary artery disease, and Type 2 diabetes mellitus[13]. Obesity has also been shown to be

associated with the incidence of several cancers such as colon, rectal, breast, ovarian, cervical, as well as prostate cancer and leukaemia[14].

1.1 Adipokines and Adipocytes:

Adipose tissue can be classified as brown adipose tissue (BAT), or white adipose tissue (WAT). WAT is primarily comprised of adipocytes, preadipocytes, macrophages, endothelial cells, fibroblasts and leukocytes. Brown adipocytes are largely responsible for generating heat in neonates, which comprise ~5% of the total adipose mass[15]. Accordingly, BAT is abundantly filled with mitochondria, which produce heat as a normal by-product of ATP production. Throughout the progression into adulthood, the number of brown adipocytes diminishes as thermoregulation is maintained through other mechanisms, such as shivering. White adipocytes were previously thought of as merely a storage warehouse for excess energy, being stored as triglycerides and released and used in times of energy shortage (fewer calories consumed than needed). For example if energy intake exceeds energy expenditure by just 5% every day, it can result in an increase in 5 Kg of fat over 1 year[16], and if this trend continues for many years it can lead to becoming overweight or obese. However, in 1994 the idea for WAT being strictly a passive storage system was discarded for it being a highly dynamic organ with the discovery of Leptin (LEP)[17]. Today over 50 adipokines[18] (proteins produced & secreted by adipose tissue) have been discovered, that can act locally in an autocrine/paracrine fashion or systemically as an endocrine hormone (**Figure 1**)[19]. They can exert their biological actions on target cells by classical ligand-receptor endocrine mechanisms. Two of these adipokines, LEP and adiponectin (ADIPO), have received much of the attention.

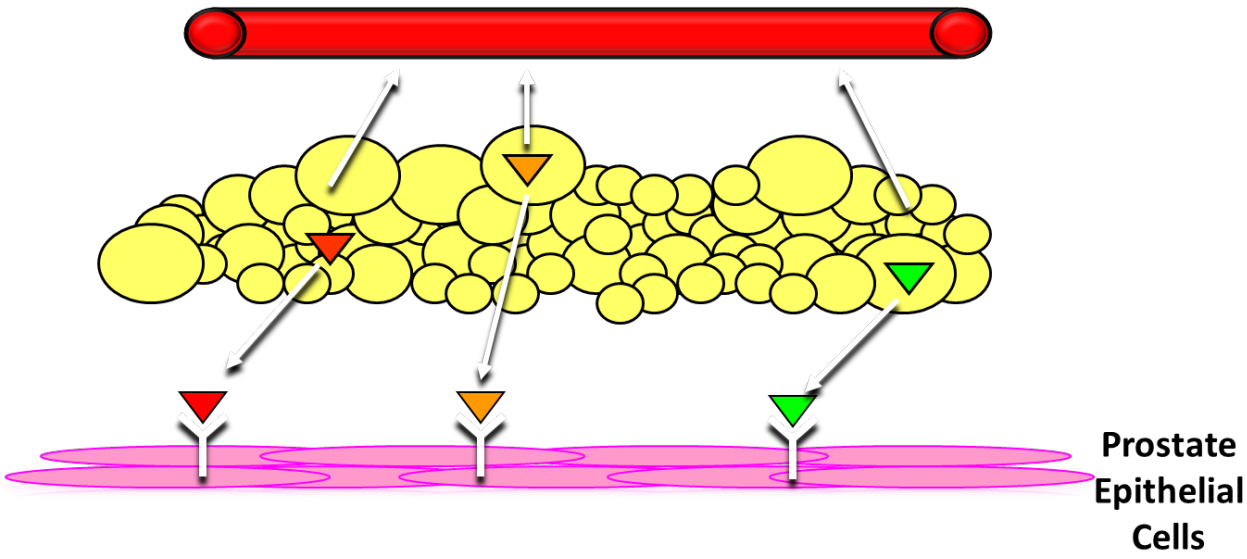

Figure 1 — Basic schematic of adipokines and their paracrine and endocrine environments.
Adipokines can travel to their target organs via the circulatory system and/or migrate to surrounding tissue to induce their various endocrine and paracrine effects.

1.2 Leptin:

LEP plays an important role in homeostatic regulation and in hunger/satiety. LEP is predominantly produced by WAT and is a 167 amino-acid protein with a molecular weight of 16 kDa[20]. The protein belongs to a family of cytokines with four long helices in their structure. LEP is encoded by the ob (LEP) gene on chromosome 7 in humans[21]. LEP carries its function through specific receptors, and there are 6 isoforms of this receptor that differ in their intra-cellular C-terminal sequences[22]. One is a long isoform LepRb, while the others have shorter C-terminal sequences[22, 23]. Although there are several isoforms, only the LepRb full-length isoform contains the distinct transmembrane subunits necessary to induce the intracellular signalling cascade from external cues[24]. Studies on mice have found that this LepRb full-length isoform is most important for transmitting the LEP signal to the cells and is located predominantly in the hypothalamus and not in most other tissues[25], while other isoforms are found throughout the

body[26]. LEP functions by binding to the receptor to cause receptor dimerization leading to the activation of the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway[27].

LEP acts through its receptor to inhibit eating rather than to initiate the feeling of fullness known as satiety. In rodents LEP production is stimulated and remains elevated for several hours after feeding, and it was also found that administration of leptin greatly reduced food intake[28]. However, this does not seem to be the case in humans as acute changes in food intake are not accompanied by short-term increases in plasma leptin levels[16], but LEP is still involved in the regulation of adipose tissue and body weight. When LEP binds to its receptors it exerts its effects on two populations of hypothalamic neuropeptides. On one population, LEP reduces the expression of orexigenic (feeding inducing) peptides, while on the other it increases the expression of anorexigenic genes (loss of appetite)[16]. Thus, LEP has an effect on controlling body weight through these two antagonistic pathways.

The circulating LEP concentration is proportional to total adipose tissue mass (**Figure 2**)[29,30], meaning that the more obese an individual is the higher the amount of circulating LEP is. Conversely, decreased serum levels of LEP are circulating in lean individuals. Serum LEP levels also appear to be 3 times higher in women compared to men[31]. The problem with obesity in rodents as well as humans is that LEP keeps on increasing as obesity increases but it no longer has the same weight control effects as the person or rodent become LEP resistant. Rodents with dietary-induced obesity (DIO) are hyperleptinemic, but become resistant to effects of exogenous LEP administration. However they do respond to LEP administration via the intracerebroventicular route, indicating that a defect in the blood-brain barrier plays a role in LEP resistance for animals[32,33]. When DIO rodents were given LEP directly into the CNS, it was

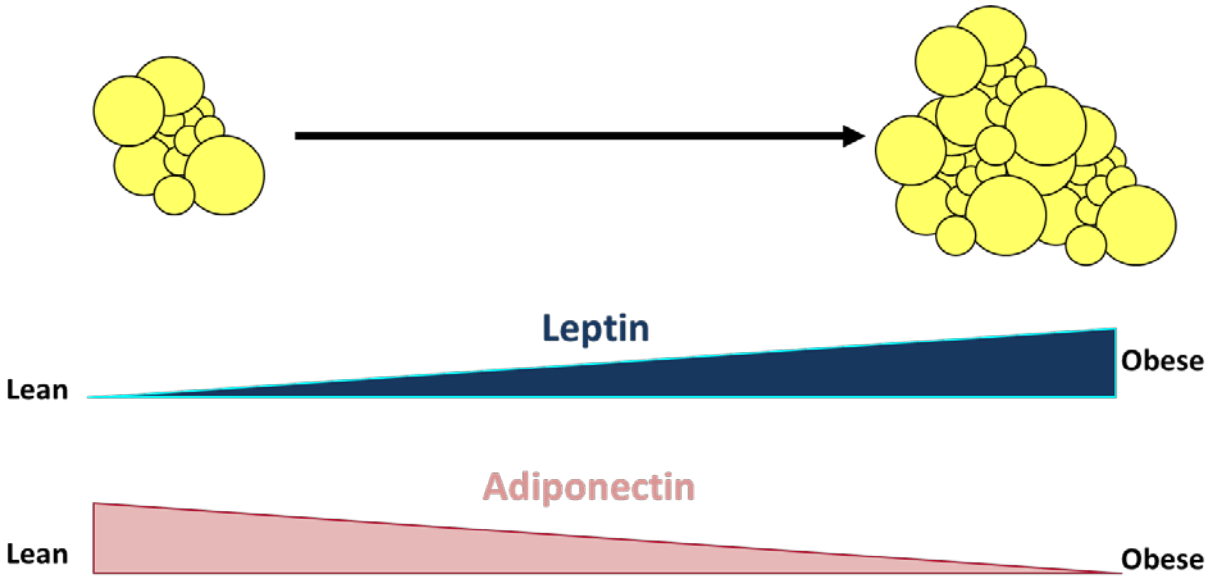

Figure 2 — Relationship of LEP and ADIPO serum levels with adiposity.
LEP production is proportional to adiposity while ADIPO production is inversely proportional to

marked by weight loss and reduced food intake also[33]. Obese humans like DIO rodents also demonstrate LEP resistance but not only are they resistant to endogenous LEP but also to exogenous LEP administration[34]. However, LEP has not yet been injected directly into the CNS of human subjects. This LEP resistance that is brought on by obesity can be potentially explained by the limitations of the blood-brain-barrier LEP transport system. The transport of LEP into the cerebrospinal fluid (CSF) is mediated by a short form of the LEP receptor[35], which is saturable[32,36]. This receptor is not as efficient in obese individuals as it is in lean individuals. The transporter is saturated at a plasma LEP concentration of about 25 ng/ml[32,36], above which there is no further increase in CSF LEP concentration despite the high value of plasma LEP concentration. Since the excess LEP from obese individuals isn't able to enter the CSF and is thus unable to trigger the hypothalamus to trigger an increased response, which could account for LEP resistance.

LEP does not exert all of its effects through the hypothalamic cells, but through other tissues that have the LEP long form receptor; such as skeletal muscle, liver, adipose tissue as

well and pancreatic β cells[16]. The product of LEP binding to the receptor in tissue such as muscle is the stimulation of lipid oxidation, which would promote insulin sensitivity[37,38].

1.3 Adiponectin:

A year after LEP was discovered ADIPO was discovered in murine adipocytes[39], and in 1996 it was discovered in human adipocytes[40]. The major functions of ADIPO are anti-atherogenic, anti-inflammatory and insulin-sensitizing effects[41]. ADIPO is a 244 amino acid protein with a molecular weight of 30 kDa that is almost exclusively produced in WAT. However, there are sources that suggest that BAT also produced some ADIPO[42]. ADIPO has also been found to be produced by cardiomyocytes in humans and mice[43]. The gene coding for ADIPO is located on chromosome 3 in humans. ADIPO exists largely as low molecular weight (LMW) trimers, medium molecular weight (MMW) hexamers and high molecular weight (HMW) nonamers[44]. Full length ADIPO can also be cleaved to produce globular ADIPO (gADIPO). ADIPO is a protein hormone, and exerts multiple biological effects throughout the body mediated by the specific receptors AdipoR1, AdipoR2, and T-cadherin[45]. AdipoR1 is expressed ubiquitously but at highest levels in striated muscles while AdipoR2 is most highly expressed in the liver. Both AdipoR1 and AdipoR2, like G-protein coupled receptors contain seven transmembrane domains[46], except that they have C-terminal domain on the extracellular side and the N-terminal domain intracellular, which is the opposite of G-protein coupled receptors[47]. T-cadherin has been shown to possibly play a very minor role in ADIPO signaling, whereas AdipoR1 and AdipoR2 mediate the vast majority of responses to ADIPO[46]. AdipoR1 and AdipoR2 mediate their responses in different manner. AdipoR1 works via activation of AMP-activated protein kinase (AMPK) while AdipoR2 works through activation of peroxisome

proliferator-activated receptor (PPARα) (**Figure 3**)[46, 47]. Full length ADIPO acts on both skeletal muscle and the liver by stimulating the phosphorylation and subsequent activation of AMPK, while gADIPO is only capable of doing this in skeletal muscle[48]. When AMPK activation is blocked the fatty-acid combustion along with glucose utilization is inhibited, suggesting that ADIPO works through and AMPK dependent manner[48]. ADIPO is one of the most abundant adipokines and circulates in the body at 0.05% of total serum proteins[39]. Like LEP, ADIPO also has higher serum concentration in women[49].

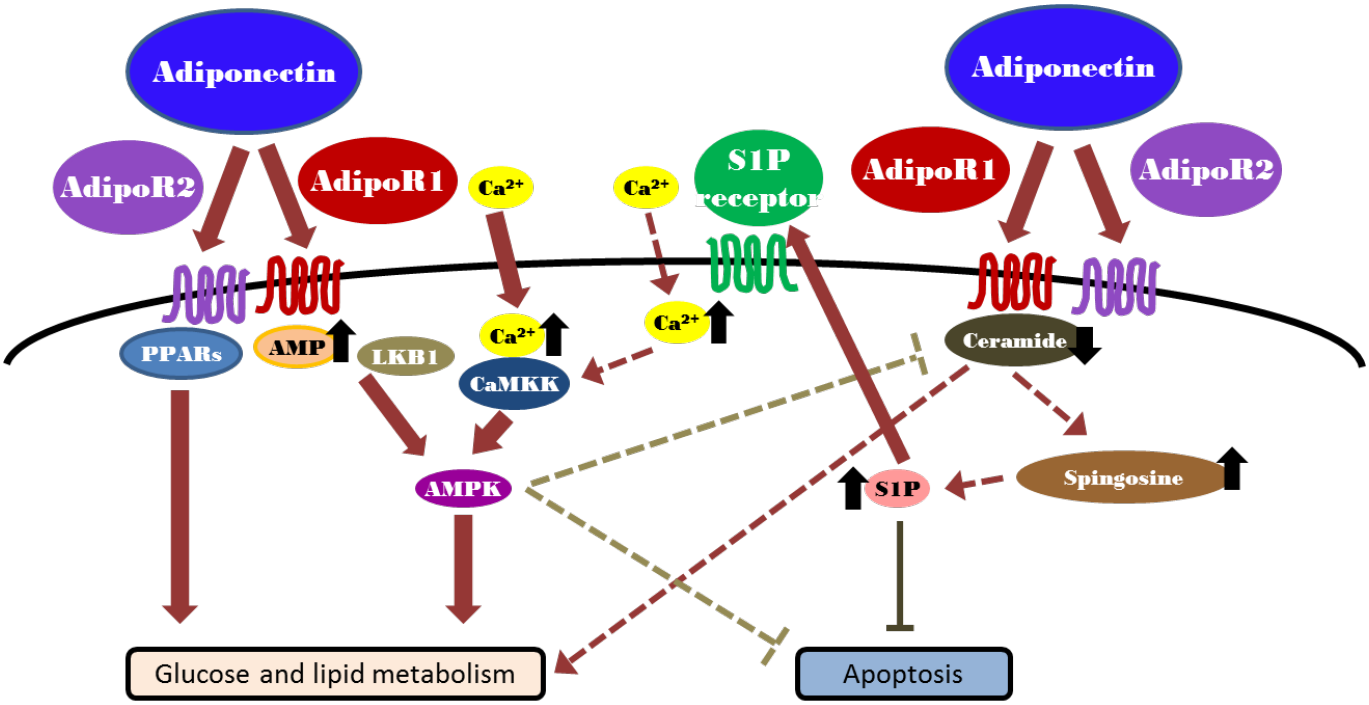

Figure 3 — AdipoR1 and AdipoR2 signaling pathway.
AdipoR1 and AdipoR2 appear to be integral membrane proteins; the N-terminus is internal and the C-terminus is external; opposite to the topology of all reported G protein–coupled receptors (GPCRs). AdipoR1 is abundantly expressed in liver, skeletal muscle, macrophages, hypothalamus and so on, while AdipoR2 is expressed in liver, white adipose tissue (WAT), vasculature and so on. AdipoR1 and AdipoR2 serve as receptors for adiponectin and mediate increased AMP-activated kinase (AMPK) and PPARs activities, thereby regulating glucose and lipid metabolism. Both Ca2þ/CaMKKb and AMP/LKB1 are necessary for adiponectin-induced full AMPK activation. Adapted from Yamauchi, T. 2014[54].

Recently, it has been realised that HMW ADIPO is the active form[50, 51], and an increase in HWM compared to total ADIPO is associated with improvements in insulin sensitivity, as shown in individuals taking thiazolidinedione for the treatment of type 2 diabetes[50]. Increased levels of total ADIPO have also been shown to increase insulin sensitivity while decreased ADIPO levels are associated with insulin resistance[52]. In a study with mice that were fed a high fat diet, ADIPO levels were observed to drop, and when ADIPO was administered it resulted in significant improvement in insulin resistance that was seen as a result of high fat feeding[53, 54]. Thus, ADIPO is described as an insulin-sensitizing adipokine. It has also been shown that a small increase in circulating ADIPO levels causes a transient decrease in basal blood glucose levels. This is believed to be the result of ADIPO sensitizing the body to insulin. In addition, ADIPOR causes an increase in fatty-acid oxidation in muscle, decreases plasma glucose and leads to overall weight loss[55-57].

The expression of ADIPO receptors is negatively regulated by insulin through the activation of phosphoinositide-3-kinase (PI3K) and inactivation of FoxO1[58]. So an inverse correlation exists between plasma insulin levels and ADIPO receptor expression[59]. Although ADIPO is produced predominately by adipocytes it has an inverse relationship with total adiposity, opposite to LEP adiposity relationship (**Figure 2**). As an individual becomes more obese they have lower levels of circulating ADIPO and as person becomes leaner they have higher circulating levels of ADIPO[52]. Thus obesity negatively acts on ADIPO in two ways. Firstly there is a decrease in ADIPO levels associated with obesity and secondly through reduced expression of ADIPO receptors, which in turn leads to reduced ADIPO sensitivity and increased insulin resistance, thus perpetuating the cycle. Also, it has been found that low levels of

circulating plasma ADIPO or hypoadiponectinemia is in itself a marker for insulin resistance and significantly related to type 2 diabetes (T2D) development[60-62].

ADIPO has also been shown to have anti-inflamitroy and anti-atherogenic properties as it is seen to accumulate in damaged vascular walls and beneficially modulate the endothelial inflammatory response to vascular injury[63].

Adipokines such as LEP and ADIPO have several differing actions on cell proliferation and cell cycle regulation.

2.0 The Cell Cycle:

The cell cycle refers to the overall process in which a cell grows, replicates its DNA and then divides into two identical daughter cells, each with an exact copy of the original DNA. The mammalian cell cycle is very intricate and tightly regulated, with several checkpoints and redundancies. The cell cycle can be divided into two major phases, interphase and mitosis[64]. Interphase has 3 sub stages that include two growth phases (G_1, G_2), with G_1 preceding S phase and G_2 succeeding it. In G_1, the cell begins to grow and increases protein production in preparation for DNA replication, which occurs in S phase. This is followed by the second growth phase, G_2. After passing through the different phases of interphase a cell is ready to divide by mitosis in which two equal daughter cells well be produced. Between every phase of the cell cycle there are critical check points that are called Restriction Points (RP) or commonly referred to as the 'point of no return'[64]. These RPs, allow a cell to perform a self-assessment to determine if the previous stage was sufficient to exclude potential termination of replication or cell death. This entire process is unidirectional, and as such once a cell reaches a RP it either passes "assessment" and continues or if it fails it is programmed to stop dividing and possibly undergo programmed cell death. One of the most vital transitions is from G_1 to S, as this is where the cell

commits to replicating its DNA. Cells that are not dividing are termed quiescent (non-proliferating) and enter G_0 or a resting state of the cell cycle[64].

Even this simplified representation of the cell cycle reveals the extreme complexity in which the cell cycle is regulated (**Figure 4**). If any mutations or errors occur in a cell they will be passed on to future daughter cells if not corrected before DNA replication or division. To ensure that the cell transitions seamlessly from the stages and beyond the RPs, several proteins are used as regulators of the cell cycle providing either stimulatory or inhibitory effects.

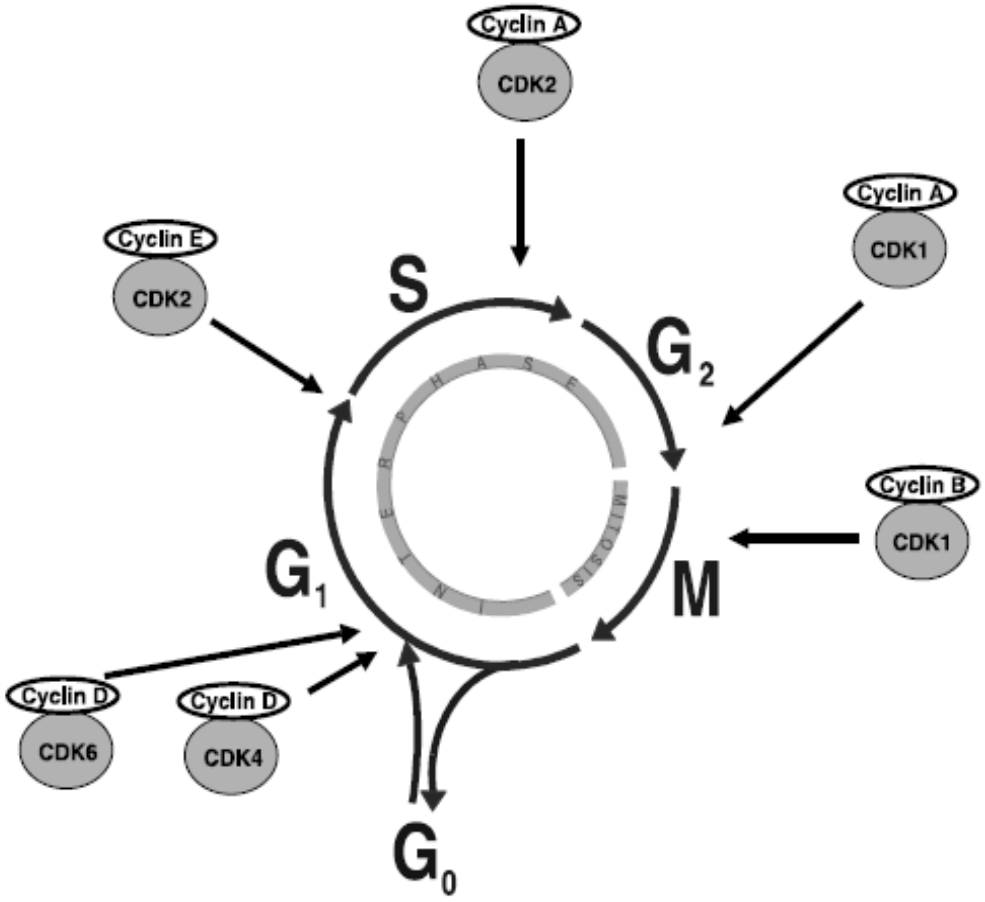

Figure 4 — The stages of the cell cycle.
The site of activity of regulatory CDK/cyclin complexes is also indicated[64].

Of these proteins the major cell regulators are the cyclin-dependent kinases (CDKs), that requires the binding of a specific cyclin to become active[65,66]. Once activated the CDK/cyclin complex regulates many downstream signaling pathways in cell cycle progression (**Figure 4)**. Cylin E and CDK2 form a complex that is responsible for cell cycle progression from G_1 to S phase[67]. CDK inhibitors counteract the stimulatory effects of the CDK/cyclin complexes[68], by either 1) preventing CDK/cyclin complex from forming, or 2) by binding to the formed complex and inactivating it, thus preventing the complex from affecting the downstream targets. Depending on their function this group of cell cycle inhibitors can be divided into two distinct families, the INK4 (p15, p16, p18, p19) family and the Cip/Kip family (p21, p27, p57)[68,69].

As shown in figure 1, the predominate G1 cyclin/cdk complexes are cyclin D/cdk4/6 and cyclin E/cdk2. Following the activation of these complexes there is a subsequent activation of the cyclin A/cdk2 in S phase and cyclin A/cdk1 and cyclin B/cdk1 in G2 and M phases[70]. Although cyclin D is expressed periodically unlike the other cyclins it is synthesized as long as the growth factor stimulation continues[71].

In terms of cell cycle regulation, the sole target phosphorylated by cyclin D/cdk4/6 is the retinoblastoma tumor suppressor (Rb). During early G1, Rb becomes phosphorylated and inactivated by cyclin D/cdk4/6 complexes, subsequently releasing the transcription factor E2F-1. This positively regulates the transcription of genes whose products are required for the progression into S phase such as cyclin E and cdc25[72]. Once a cell reaches full activation of cyclin E/cdk2 in late G1, the cell has passed the restriction point. Following this, the cell is committed to enter the cell next phase of the cell cycle and can't return back to G1 until a full cell cycle is completed. Importantly, limiting the amount of active cyclin E/CDK2 available keeps cells inactive until a mitogenic signal intervenes. Cyclin E expression is dependent on its

regulation by E2F transcription factors once they have been released from Rb. When a cell is in the G0 quiescent state, Rb is in a hypophosphorylated state and bound to E2F[64] inhibiting transcription of cyclin E mRNA.

Initially, these mitogenic signals induce the expression of cyclin D and cdk 4/6 which then forms an active kinase state with p27 in order to phosphorylate Rb. This phosphorylation causes the dissociation of E2F from Rb which then transcribes cyclin E allowing for the formation of the active kinase cyclin E/cdk2[64]. This newly formed cyclin E/cdk2 then works through a positive feedback loop to cause Rb to become hyperphosphorylated thus causing E2F to fully activate its target genes and induce the transition into S phase and DNA replication.

As previously mentioned p27 is a key regulator of the G1-S phase transition. It serves to prevent premature activation of cyclin E/cdk2 in early G1 and also helps with the assembly and activation of cyclin D/cdk4/6 in late G1[73]. Therefore p27 can be thought as a positive regulator of G1 progression by this assembly and nuclear import of cyclin D/cdk4/6[73]. It is now believed that the balance between the levels of cyclin D1 and p27 is the main regulating mechanism for controlling proliferation of cells instead of their absolute levels within the cells[74].

The discovery of p27 was first shown by its inhibition of cyclin E/cdk2 and cyclin A/cdk2 in cells arrested by TGF-β and contact inhibition[75-78]. Specifically, p27 was shown to exhibit its inhibitory effect on cyclin E/cdk2 by binding to the catalytic cleft of the complex, preventing it from phosphorylating Rb[79]. Mitogenic growth factor signals have been shown to cause a loss of p27 levels and activity. In contrast p27 has been shown to increase in response to differentiation signals[80-82].

The mRNA expression of p27 does not change through the cell cycle but instead the protein content of p27 is regulated by stabilizing phosphorylation in G_0/early G_1 and inactivating phosphorylation in late G_1/early S phase[80, 83].

Phosphorylation of p27 can result in loss of its inhibitory activity or degradation, however not all phosphorylation sites on p27 cause ubiquitin mediated degradation and/or nuclear export, as some facilitate p27 effects on cell cycle arrest and protect it from these events[84]. One of the important phosphorylation events occurs on the threonine 187 residue (T187) by the "newly-activated" cyclin E/CDK2 holoenzyme[85]. Phosphorylation at this particular site targets p27 to the Skp2 multi-protein ring finger ubiquitin (Ub) nuclear degradation complex and accounts for nearly 80% of total p27 protein degradation[86]. While evidently a predominant mode of p27 inactivation, protein quantification of Skp2 revealed that p27 levels decline rapidly in mid G1, prior to the rise of Skp2 expression seen in late G1[87, 88]. This observation suggested the presence of a novel proteolytic pathway involved in p27 degradation. Subsequent research has since verified the presence of such a mechanism albeit within a different sub-cellular location. Phosphorylation of the serine 10 residue (S10; likely by hKIS or AKT) serves to translocate p27 out of the nucleus and into the cytoplasm[87]. Here, it can be recognized by KPC1 and KPC2, members of the KIP1-ubiquitilaytion promoting complex that target p27 for early G1 Ub-mediated degradation[88].

Another phosphorylation event occurs in the cytoplasm on the threonine-157 residue (T157) of newly translated p27 protein by AKT[87]. Once phosphorylated, the family of 14-3-3 proteins interacts with $p27^{T157}$ and sequesters it within the cytoplasm where it can no longer inhibit cyclin E/cdk2, which will allow for the transcription of S-phase dependent genes[89].

A significant stabilizing phosphorylation to p27 is caused by AMP-activated protein kinase (AMPK) on threonine 198 (T198)[90,91]. This stabilizing phosphorylation is important in quiescence and early G1 as this T198 phosphorylation keeps p27 levels high and facilitates inhibition of cyclin E/cdk2 leading to cell cycle arrest. Liang et al. (2007) discovered that in breast cancer cells, activated AMPK (pAMPK) directly phosphorylated p27 on T198 ($p27^{T198}$)[90]. This then causes cell cycle arrest by increasing the stability of the protein. Also interesting to note is that Rattan et al. (2005) demonstrated that AICAR; an AMP analog which can indirectly up-regulate AMPK through its upstream kinase, liver kinase B1 (LKB1), increases pAMPK, stabilizes p27 and p53. AICAR also inhibited the AKT pathway through its upstream activator PI3K. Phosphorylation on T198 increases total p27 protein half-life when compared to T198 mutated p27 cells when activated with AICAR[90]. Thus, this stabilizing phosphorylation on p27 is extremely important to cell cycle arrest and required for optimal cell cycle progression and may play a role in cancer development/progression.

When there are fundamental alterations in cell cycle regulation this leads to uncontrolled cell proliferation. The loss of cell cycle regulation is mainly due to mutations in the cell that affect either proto-oncogenes, or tumor suppressor genes[64]. This rapid and uncontrolled cell proliferation that has the potential for metastasis is classified as cancer.

3.0 Prostate Cancer:

The prostate is an exocrine organ in males that is part of the reproductive system. The main function of the prostate is to produce and expel an alkaline fluid comprising 20-30% of semen volume that neutralizes the acidity of the vaginal tract. Prostate cancer (PCa) is screened for in the general population by using one of two methods. The first being the digital rectal examination (DRE) in which a physician inserts a gloved lubricated finger into the rectum of the patient and exams the prostate through the rectum, for irregular shape, size and texture. The second being prostate specific antigen (PSA) testing, in which the serum PSA levels are measured to determine if the levels are beyond the normal range. There is some controversy in the field regarding the use of PSA to screen for PCa. Some men with PCa might not have elevated levels of PSA which is false negative; which occours 10% of the time, and at other times some men might have elevated PSA levels without having or ever developing PCa, known as a false positive, which is a common occourrence. Only 1 in 4 abnormal PSA levels is due to PCa[92]. Another disadvantage of PSA screening is that it can detect PCa that may not pose a serious health risk to the individual, yet due to the detection of elevated PSA levels the man will undergo a biopsy and potentially treatment, even though the cancer would not have affected the individuals life. Recent research has shown that 50% of cancers that are detected by the PSA test may never need to be treated. The majority of these men go on to have treatment, which is unnecessary in most cases[92]. PSA screening is not recommended on a population wide bases, but mainly recommended to individuals at high risk for PCa and should be discussed with their doctor to determine if PSA screening is right for them[92]. This makes the efficacy of PSA screening in reducing overall mortality from PCa a source of great controversy[93,94], which stems from over diagnosis and lack of specificity of PSA in discriminating between PCa and benign

prostatic hyperplasia[94]. While PSA has been shown to correlate very well with tumor volumes[95], it is unable to predict with certainty the biological aggressiveness of the disease or its prognosis. Thus there is still a need to develop a non-invasive test using biomarkers to identify clinically relevant PCa.

Once diagnosed with PCa there are several ways to manage PCa, among the most common are androgen deprivation therapy (ADT), radical prostatectomy, external beam radiation therapy or chemotherapy or a combination of these methods. Androgen receptor positive PCa can be initially managed through ADT, which is the chemical equivalence of physical castration. ADT can be accomplished through several methods, such Orchiectomy (surgical castration), where the testicles are surgically removed or through chemical castration using luteinizing hormone-releasing hormone (LHRH) analogs, which reduces the testosterone produced in the testicles. Another option for ADT is using anti-androgens, which bind to the androgen receptors prevents the androgens from having the desired effect on PCa. All of these treatment options reduce the overall levels of androgens available to enhance PCa growth and metastasis. ADT is used as a first line of treatment for metastatic PCa[96]. The cancer initially responds to ADT and regresses but after time the cancerous cells can become androgen-independent (also known as castration-resistant or hormone-refractory PCa) and are no longer responsive to ADT, making the tumour more aggressive[97]. As a result of these treatments some men might become impotent and depressed along with other side effects due to ADT, and this poses yet another question regarding the efficacy of PSA screening.

PCa will account for 24% of newly diagnosed cancers in the United States, and will account for 10% of cancer related deaths in men[92]. According to the Canadian Cancer Society, one in 8 men will develop PCa during his lifetime (the risk is highest after age 60) and one in 28

will die of it. Also in Canada PCa was the most common diagnosed cancer excluding melanomas[98]. Despite much research on PCa, its cause still remains unclear. However several risk factors have been identified such as age, race/ethnicity, nationality, family history, genetics and diet[99, 100]. Obesity has also been shown to be a risk factor for PCa[101]. A recent study has shown visceral fat accumulation; quantified by computerized tomography (CT) is a specific risk factor for PCa[102]. However obesity has a greater association with PCa mortality as well as higher grade and more aggressive tumours[103].

A geographic correlation exists between areas of high PCa and obesity incidence. Not surprisingly PCa incidence is highest in North America and Western Europe. Specifically England and Wales, are associated with the highest obesity rates, whereas Asian countries have the lowest incidence[104,105]. Recently there has been a rapid rise in PCa incidence in Asian countries[105] that is mirrored by the explosive obesity trend in Asian regions such as China. The search for the molecular link between obesity and PCa as well as many other cancers has led to the focus on adipokines as cancer growth regulatory factors, particularly LEP and ADIPO.

A positive correlation between circulating LEP levels and the risk of large-volume prostatic tumours has been found[106]. There is also evidence that serum leptin levels are correlated with measures of prostate cancer risk including PSA and Gleason score in patients with PCa[107]. Gleason score is the most common grading method for PCa, starting at 1 and going to 5. A score below 3 is rarely used for PCa because the tissue looks and acts like normal cells, while higher scores reflect a worse prognosis and metastisis[92]. *In vitro* studies support the proliferating effect of LEP on androgen independent PCa cell lines[108], which mimic castration-resistant PCa. High LEP concentration is predictive of high grade disease and more advanced tumors[109]. Since

obesity is associated with increased PCa incidence, as well as more aggressive tumours, it is suggestive that LEP is a potential mediator of this because it follows the same trend.

ADIPO has been found to be negatively correlated with adiposity. Lower ADIPO levels were found in patients with PCa as opposed to healthy controls and patients with benign prostatic hyperplasia [110]. In that same study it was also found that ADIPO was negatively correlated with tumour grade and Gleason scores such that lower ADIPO levels were associated with higher grade tumours and higher Gleason score. Since obesity is associated with increased LEP levels and decreased ADIPO levels, obesity correlates negatively with PCa prognosis.

4.0 Metformin:

Metformin (MET) is an oral medication for T2D that is used to reduce blood glucose levels in obese or overweight people with normal kidney function[111]. It was initially developed in the 1920s as a by-product of the synthesis of *N*,*N*-dimethylguanidine[112], but interest in MET died down and was later rediscovered and used for the treatment of diabetes. MET is the most widely used oral anti-diabetic medication for T2D, due to its clinical effectiveness and low toxicity profile [112]. In 2010, 48 million prescriptions of MET and its generic formulations were filled in the United States.

Obesity is a well-recognized risk factor for T2D that is also a risk factor for cancer itself[113, 114]. Certain types of cancers are more common in individuals with diabetes, and diabetes is also associated with reduced survival after development of cancer[115]. PCa is one of the cancers that men with T2D appear to be at a higher risk for, with a health risk of 1.19 compared to those men without T2D[116]. However, some studies provide contradicting evidence in which T2D is reported as being protective of PCa[117]. Some researchers suggest that the protective effect is due to the reduced testosterone production in diabetics[118,119], while other attribute it to the medication that the individuals are on for management of T2D [120]. Nonetheless with PCa the chances of dying from the disease are increased with T2D[121].

It has been found that patients with T2D on MET have a lower risk of developing cancer and lower mortality from cancer as opposed to patients on other glucose lowering therapies[122-126]. In preclinical studies that utilized MET *in vivo* it was found to decrease PCa cell line survival by up to 50%, while reducing normal prostate epithelial cells by only 20%, which suggests that MET selectively targets the rapidly dividing PCa cells while leaving the healthy cells relatively

undisturbed[127]. Several different pathways have been suggested to account for the anti-cancerous effects of MET which include the inhibition of mammalian target of rapamycin (mTOR) [128], and by the activation of the AMPK in some cancers[129, 130] while also being able to exert its anti-cancerous effects in an AMPK independent pathway in some cell lines[127, 131129, 130]. MET seems to be a double edged sword in the fight against T2D and cancer simultaneously and thus a promising drug for therapy.

As mentioned earlier PCa is initially responsive to androgens, but later on becomes hormone refractory or androgen independent. To mimic these conditions *in vivo* 3 different cell lines are often utilized. LNCaP cell which are androgen receptor positive represent hormone responsive PCa, and PC3 and DUI45 are androgen receptor negative and mimic hormone refractory PCa. *In vivo* studies using LEP found it caused an increase in the phosphorylation of AKT at Threonine 473 in LNCaP, PC3 and DUI45 cell lines, but only very weakly in the latter 2[132]. LEP also caused a significant increase in the number of PC3 and DUI45 cells, but only a mild increase in the number of cells for LNCaPs[133]. ADIPO on the other hand has been shown to have a dose dependant growth inhibitory effect on PC3 cells *in vivo*, likely due to an increased phosphorylation of AMPK at Threonine 172 (T172)[134]. AMPK phosphorylation at T172 has also been observed in LNCaP cells in response to ADIPO treatments *in vivo*[135], and surprisingly ADIPO caused increased phosphorylation of AKT at two sites Threonine 308 and Serine 473. The results of that study suggest that ADIPO stimulates both AMPK and PI3K/AKT pathways with the later having a dominant effect on the activation mTOR in LNCaP cells. Activation of AMPK has also been observed in PC3 cells when treated with MET *in vivo*[134].

5.0 Hypothesis:

Based on the background literature we a priori hypothesized that:

1. MET will act as inhibitor of the cell cycle through the activation of AMPK in androgen receptor positive LNCaP cells as well as androgen receptor negative PC3 cells.
2. AMPK inhibition with Compound C will abolish the effects of MET.
3. Met will counteract the effects of LEP on cell cycle progression.
4. Adipose tissue from cancer and non-cancer patients will elicit cell cycle effects on androgen receptor positive LNCaP as well as androgen receptor negative PC3 cells.

6.0 Materials and methods:

6.1 Cell Lines and Cell culture. Human prostate cancer cells, LNCaP and PC3, which are androgen receptor positive and androgen receptor negative respectively, were obtained from the American Tissue type Culture Collection (ATCC, Manassas, VA). LNCaP and PC3 cells were maintained in RPMI 1640 (Wisent, St.Bruno, QC), 10% Fetal Bovine Serum (FBS, Hyclone, Thermo Fisher Scientific, Whitby, ON), 3% & 1% Antimitcotic/Antibiotic (Wisent) respectively, at 37°C and 5% CO_2.

6.2 Metformin and cell growth. LNCaP and PC3 cells were plated into 6 well plates in RPMI 1640 for 48 hours until approximately 70-80% confluence. At 48 hours the media was replenished and the cells were treated with various concentrations (1-100 nM) of 1,1-dimethylbiguanide hydrochloride (metformin, Santa Cruz Biotechnology. Santa Cruz, CA) for 24 hours, in the presence and absence of the AMPK inhibitor Compound C (CC)(10 μM, Millipore).

6.3 Cell harvesting and lyzing. The 6 well plates were aspirated and washed twice with cold Phosphate Buffered Saline (PBS, Wisent) scraped and transferred into a single centrifuge tube with 1 mL of cold PBS. Samples were then centrifuged at 2,300 g at 4^{o}C for 5 minutes. The supernatant was discarded and the pellet was then re-suspended in TENT++ (0.2% Tent [TRIS, EDTA, NaCl, and 0.2% Triton x-100]), Sigma protease inhibitor cocktail and Sigma phosphatase inhibitor cocktail 2, (Sigma, Oakville, ON). Samples were then sonicated for 5 seconds at 10% power (Microsonic Unltrasonic Cell Disruptor, VWR, USA) and centrifuged for 10 minutes at 15,700 g. The supernatant was then removed and stored at -84°C for future analyses.

6.4 Human Samples. In collaboration with The Princess Margaret Hospital and the University Health Network, male patients who were undergoing prostatectomy or invasive exploratory surgery were recruited to the study. After patient consent was received and prior to the surgery the patient's serum was collected and their weight, age, and medication were identified. The study is run in a blind manner as to not bias any of the results. During the surgery adipose tissue samples were obtained from the patients. Two samples were obtained, one from peri-prostatic fat for patients undergoing prostatectomy as well as visceral fat from the abdomen. For patients undergoing exploratory surgery or nephrectomy the samples obtained were from the visceral fat storage from the abdomen as well as the second sample from the peri-prostatic/renal fat depending on the surgery.

6.5 Conditioned Media. To generate the conditioned media (CM) human fat from 35 patients was incubated with RPMI (15 ml od RPMI/ g of minced tissue), that contained 10% FBS and 1% Antimitcotic/Antibiotic. The samples were placed in RPMI media for 30 minutes and incubated 37°C with 5% CO_2. The media was aspirated out and the fat samples removed and coarsely minced with scissors and placed in the appropriate amount of RMPI media for 1 hour and incubated at 37°C with 5% CO_2. Subsequently the RPMI media was aspirated and replenished and left for 24 hrs incubated 37°C with 5% CO_2 with periodic mixing. After 24 hous a sample of the fat was frozen and stored at -84^{o}C for future analysis, and the CM collected and stored at -84^{o}C.

6.6 Conditioned Media Treatment. PC3 cells were seeded in 6 well plates with RPMI until cells were 70-80% confluent. The media was aspirated and washed with PBS and then CM from either ACM or PCM was added and left for 24 hours. Additional plates were treated with 25 nM metformin and or 10 µM Compound C, depending on whether sufficient volume of CM was

obtained as described above. Cells grown in normal RPMI containing only 10% FBS were used as controls. All treatments were for 24 hours. Prior to collection of the cells the CM was collected and frozen at -84^{o}C for future analyses and the cells were harvested as described above.

6.7 Western Blotting. Protein concentrations were determined using a Bradford Assay (BioRad) and 25 µg of protein was loaded onto 12% SDS polyacrylamide denaturing gels (PAGE), were separated by electrophoresis and transferred onto a PVDF membrane (Bio Rad, Mississauga, ON) at 4°C overnight. Blots were stained with amido black to determine protein transfer efficiency. Membranes were blocked for 2 hours in 10% low fat milk and then washed with TBST (Tris-Buffered Saline with 0.5% Tween 20, TBST) for 4X10 min. Subsequently membranes were incubated with the appropriate specific primary antibodies (Abs) in 5% BSA at 4°C overnight. Following the primary incubation the membranes were washed with TBST for 4X10 min and then incubated with specific secondary Abs (in 5% low fat milk) for 1 hour and subsequently washed again in washed with TBST for 4X10 min. Proteins of interest were then visualized by ECL Chemiluminescent HRP Substrate (Millipore) using the Kodak In vivo FX Pro Imager to detect the chemiluminescent signal which was quantified using Carestream imaging software (version 5.0.5.31).

Proteins of interest were studied by Western blotting with primary Abs for mouse monoclonal anti-p27^{Kip1} (1:3000, BD Biosciences), rabbit polyclonal anti-p27^{T198KIP1} (1:2000, R&D Systems, Minneapolis, MN), rabbit polyclonal anti-pAKT^{T308} (1:500, Cell Signalling), rabbit polyclonal anti-AKT total (1:3000, Cell Signalling), rabbit polyclonal anti-pAMPK^{T172} (1:1000, Cell Signalling), rabbit polyclonal anti-AMPK total (1:2000, Cell Signalling), goat polyclonal anti-ADIPOR1 (1:2000, Santa Cruz Biotech), mouse monoclonal anti-Cyclin E (1:1000, AbCam Cambridge, MA), rabit polyclonal anti-COX IV (1:5000, Abcam) and mouse monoclonal anti-β-

actin (1:50000, Abcam) for 24 hrs at 4°C. Secondary Abs (1:5000, rabbit, mouse or goat, Promega, Madison, WI) or β-actin (1:20000, anti-mouse, Promega), were incubated with the membrane at room temperature in 5% low fat milk for 1 hr. All protein levels have been corrected to β-actin, to account for any errors due to uneven loading.

6.8 Statistical analyses. GraphPad's Prism 5 for windows version 5.02 was used for all statistical analysis. For MET concentration alone a 1-way ANOVA was utilized along with Tukey's post hoc test to compare the different concentrations. MET and Compound C treatments were alayzed using a two-way ANOVA was utilized in combination with bonferroni post hoc test. For MET, Compound C and LEP treatments, as well as CM treatments multiple t-tests were used to evaluate the desired comparisons. Differences were considered significant for all tests when $p < 0.05$.

7.0 Results:

7.1 LNCaP – Metformin Treatment

LNCap cells treated with 50 and 100 nM detached from the plate and demonstrated distinct morphological changes. $pAMPK^{T172}$ was increased by approximately 200% with 1nM MET treatment with a steady decline evident as MET concentration increased reaching 42 ± 16% of control at 50 nM, an 80% reduction from 1 nM MET (**Figure 5 A,B**). $pAKT^{T308}$ displayed a dramatic decrease with MET treatment, declining to levels that were 11 ± 7% of those in non-treated control cells (**Figure 5 A,C**). $pAKT^{T308}$ was always lower than $pAMPK^{T172}$ values at all concentrations of MET, as predicted. This suggests that AMPK activation may lead to AKT suppression. Predictably $p27^{T198}$ displayed a similar pattern as $pAMPK^{T172}$, reaching its lowest levels at 50 nM (**Figure 5 A,D**). p27 protein levels were also decreased by MET, reaching the lowest value at 38 ± 14% of non-treated control cells at a concentration of 100 nM (**Figure 5 A,E**). COX IV protein trended towards being decreased by MET (p=0.07) **(Figure 5 A,G**). Cyclin E protein levels were decreased by MET treatment, reaching levels that were 30 ± 8% of those seen in untreated control cells at 100 nM (**Figure 5 A,F**). AdipoR1 was also decreased by increasing concentrations of MET, reaching levels that were 40% lower than in control cells at 100 nM MET (**Figure 5 A,H**).

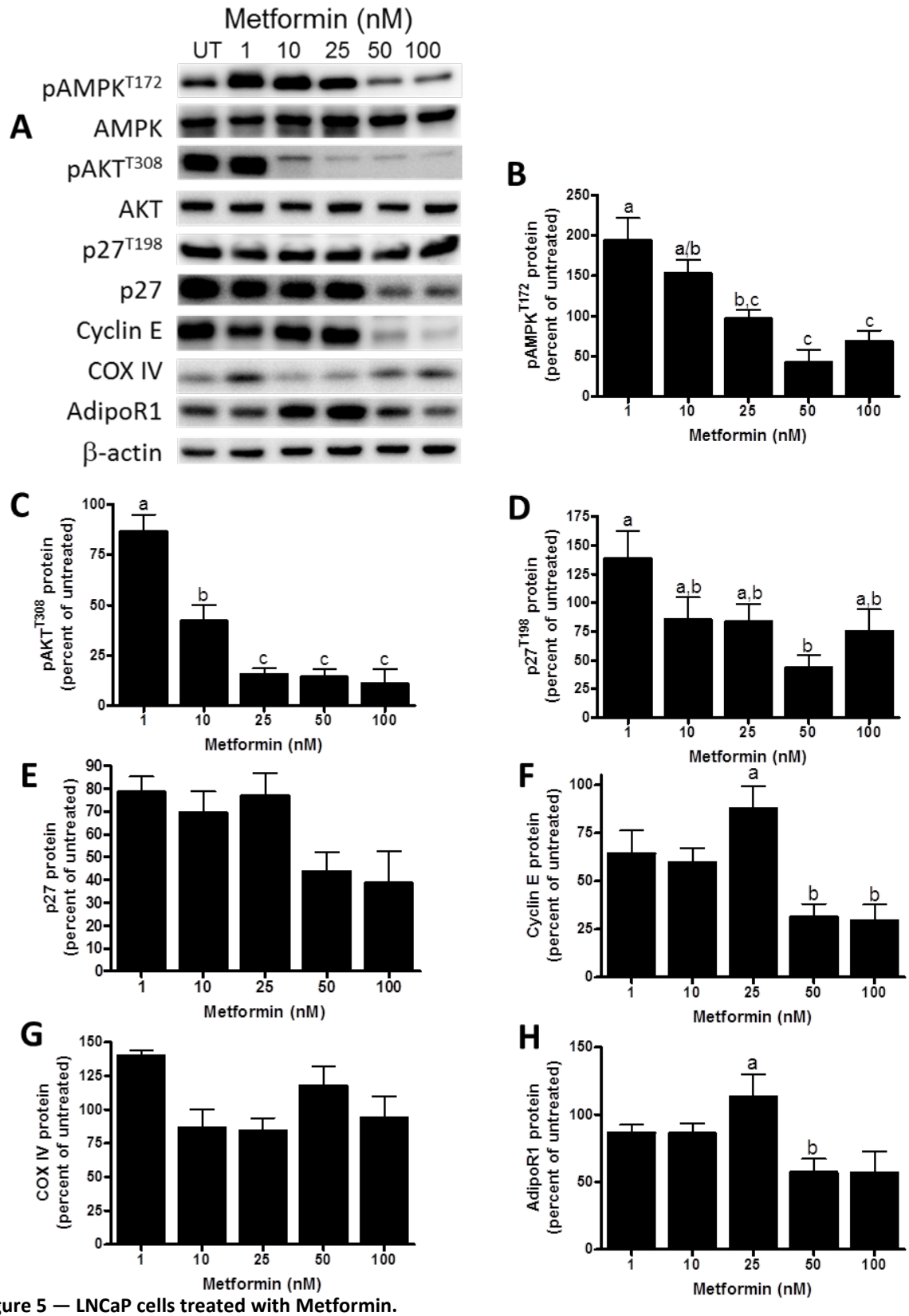

Figure 5 — LNCaP cells treated with Metformin.

(**A**) Representational blots for LNCaP cells for treated with MET, (**B-H**) graphical representation of relative optical density versus control (Mean ± SEM). Blots standardized to β-actin to correct for loading. Letters which differ represent a significant difference ($p<0.05$). n= 6-12/group

7.2 LNCaP – Metformin and Compound C Treatment

In order to determine the role of AMPK in mediating the effects of MET, experiments were conducted in the presence of the AMPK inhibitor Compound C (CC). LNCaP cells were treated with 0, 10, 25 and 50 nM of MET alone and in combination with 10 μM CC. Inhibition of AMPK by 10 μM CC had no effect on previously observed MET effects on all proteins (**Figure 6 B-H**). Interactions were observed for $pAKT^{T308}$, and COX IV (**Figure 6 C,G**). It appears that this was a result of an increase in each protein with CC in the absence of MET.

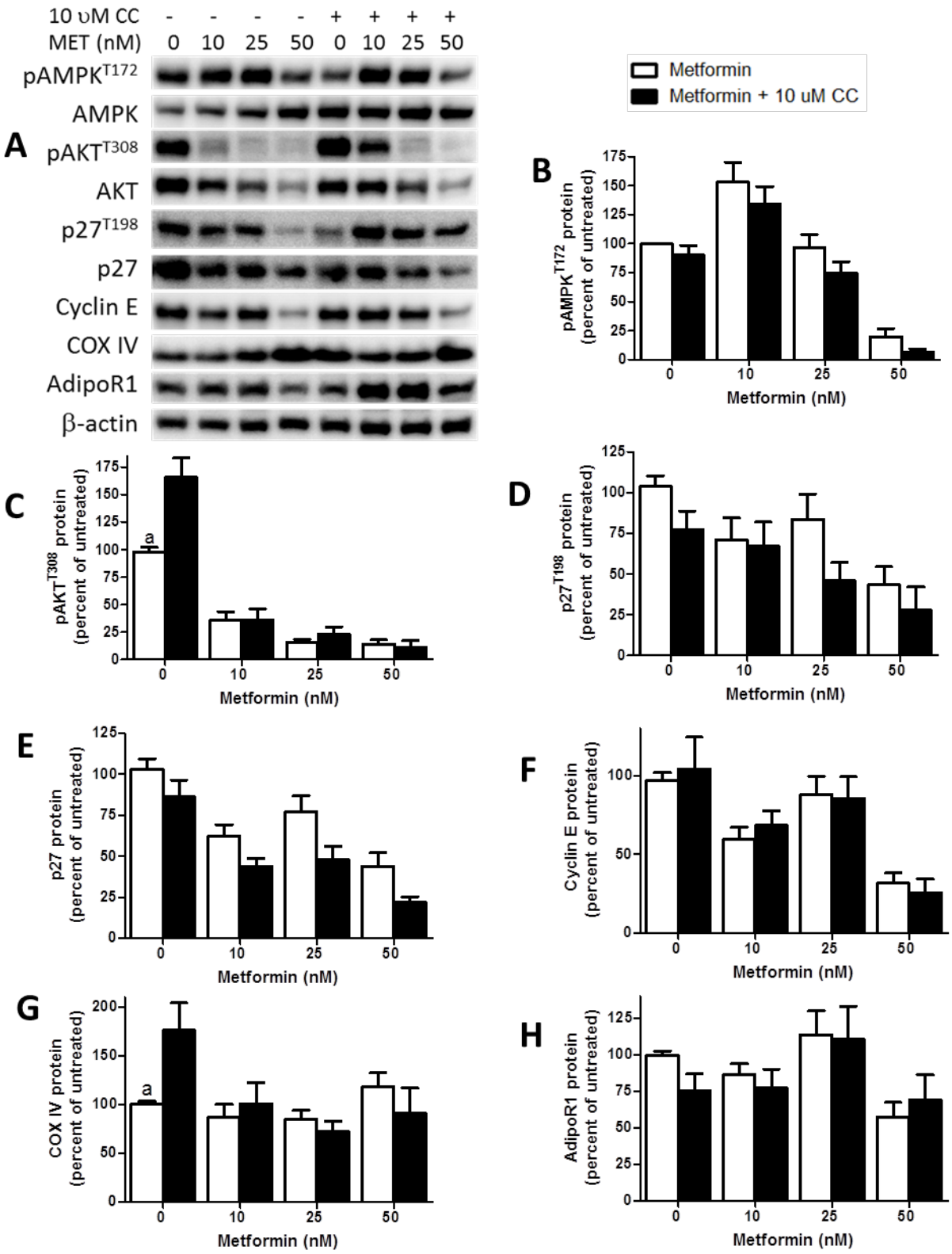

Figure 6 — LNCaP cells treated with Metformin and Compound C.

(**A**) Representational blots for LNCaP cells for treated with MET and CC, (**B-H**) graphical representation of relative optical density versus control (Mean ± SEM). Blots standardized to β-actin to correct for loading. Graphs have been rearranged for clarity. Letters which differ represent a significant difference ($p<0.05$). n= 6-12/group

7.3 LNCaP – Metformin, Leptin and Compound C Treatment

Since LEP is seen to be elevated in obesity, MET treatments were conducted in the presence of LEP to determine if MET can counteract the effects of LEP. pAMPK^{T172} was reduced to approximately 50% of levels found in untreated cells with the addition of MET in the presence of LEP. Again no effect of CC was observed. LEP and CC combined caused pAMPK^{T172} to reach its highest levels of 147 ± 13. When MET was added with LEP it caused a decrease to 22 ± 4% of untreated control cells. In the presence of CC the same results were obtained, with MET causing a decrease to 24 ± 6% (**Figure 7 A,B**). Since LEP treatments caused changes in total AKT (**Figure 7 A**) we expressed pAKT^{T308} as a function of total AKT; a similar response to pAMPK^{T172} in which the addition of MET counteracted the effects of LEP and caused a decrease in pAKT^{T308} regardless of the presence of CC (**Figure 7 C**). MET caused a decrease in p27 levels when combined with LEP in the absence of CC, however when MET and LEP were combined in the presence of CC the reduction by MET was not observed (**Figure 7 A,E**). COX IV trended (p=0.0533) towards being reduced when MET and LEP were combined in presence of CC (**Figure 7 A,G**). p27^{T198}, Cycline E, and AdipoR1 demonstrated no observable effects of treatment (**Figure 7 D,F,H**).

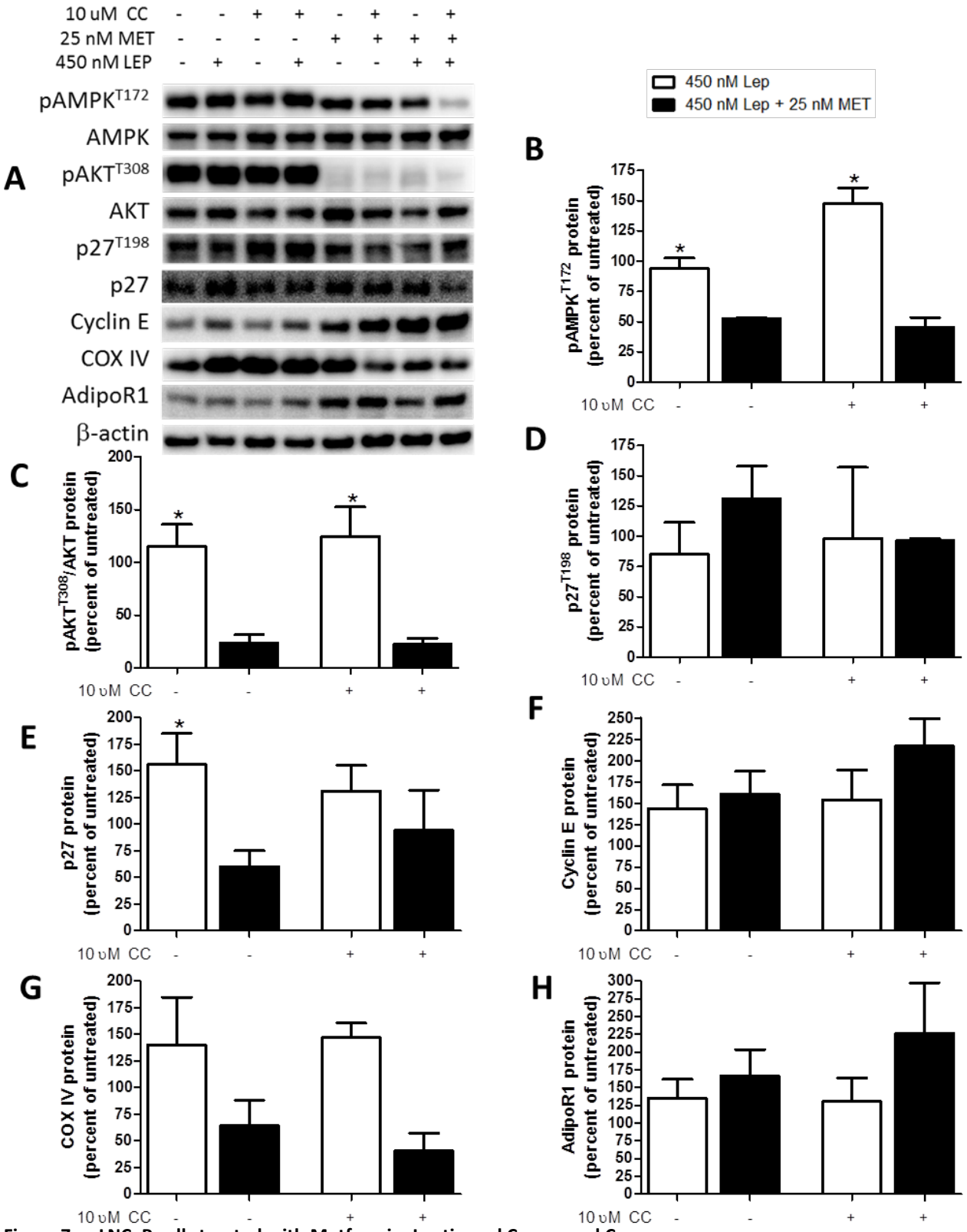

Figure 7 — LNCaP cells treated with Metformin, Leptin and Compound C.

(**A**) Representational blots for LNCaP cells for treated with MET and CC, (**B-H**) graphical representation of relative optical density versus control (Mean ± SEM). Blots standardized to β-actin to correct for loading. Graphs have been rearranged for clarity. * represents significant differences between MET treated cells in the same CC same treatment ($p<0.05$). n= 6-12/group

7.4 PC3 – Metformin Treatment

MET did not affect pAMPK^{T172} at any concentrations. Since total AMPK concentrations appeared to decrease by MET treatment (**Figure 8 A**), relative pAMPK^{T172}/AMPK was utilized for analysis. pAMPK^{T172}/AMPK increased with MET concentrations (**Figure 8 B**). Total AKT levels were also altered by MET treatment (**Figure 8 A**) and AKT activation was expressed by measuring pAKT^{T308}/AKT. pAKT^{T308}/AKT was found to decrease with MET treatment (**Figure 8 C**). p27 was reduced by MET where it reached its lowest value of 79 ± 6% of untreated control at 25 nM MET. Surprisngly 100 nM MET increased p27 to its highest value of 156 ± 21%of control (**Figure 8 A,E**). p27^{T198}, cyclin E, COX IV and AdipoR1 were found to not be affected by MET (**Figure 8 D,F-H**).

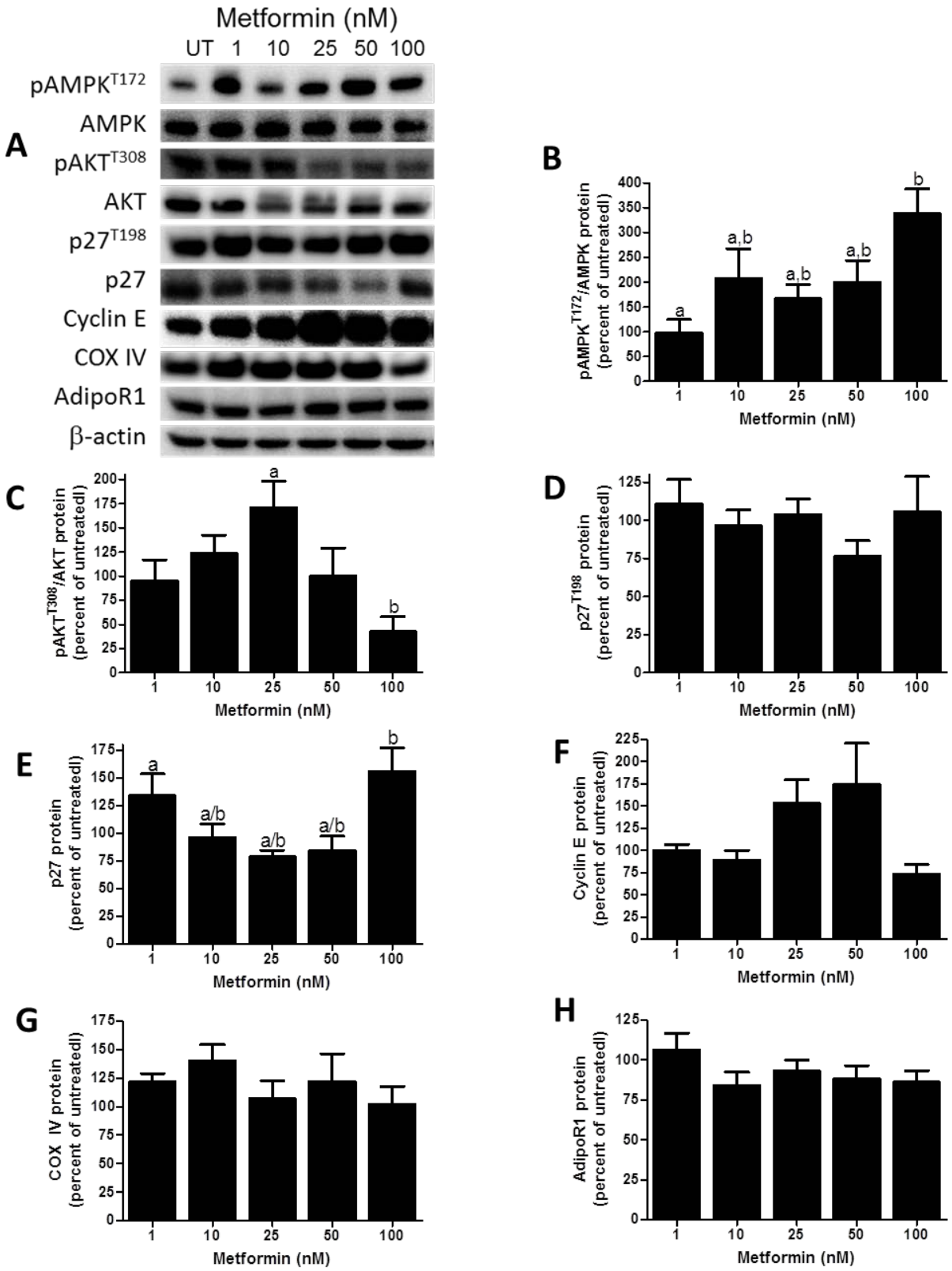

Figure 8 — PC3 cells treated with Metformin.

(**A**) Representational blots for LNCaP cells for treated with MET, (**B-H**) graphical representation of relative optical density versus control (Mean ± SEM). Blots standardized to β-actin to correct for loading. Letters which differ represent a significant difference ($p<0.05$). n= 6-12/group

7.5 PC3 – Metformin and Compound C Treatment

In order to determine the role of AMPK in mediating the effects of MET, experiments were conducted in the presence of the AMPK inhibitor Compound C (CC). PC3 cells were treated with 0, 10, 25 and 50 nM of MET alone and in combination with 10 μM CC. CC caused a decrease in $pAMPK^{T172}$ to roughly 50% of the levels in untreated control cells and remained low at all concentrations of MET (**Figure 9 A,B**). $pAKT^{T308}$ in general was increased by CC especially at higher concentrations of 25 and 50 nM MET when combined with CC (**Figure 9 A,C**). It was the only protein that showed an interaction between CC and MET. CC had no effect on p27 ^{T198}, p27, AdipoR1, COX IV and cyclin E (**Figure 9 D-G**). COX IV and AMPK were undetectable with 10 nM MET and CC treatment (**Figure 9 A,G**).

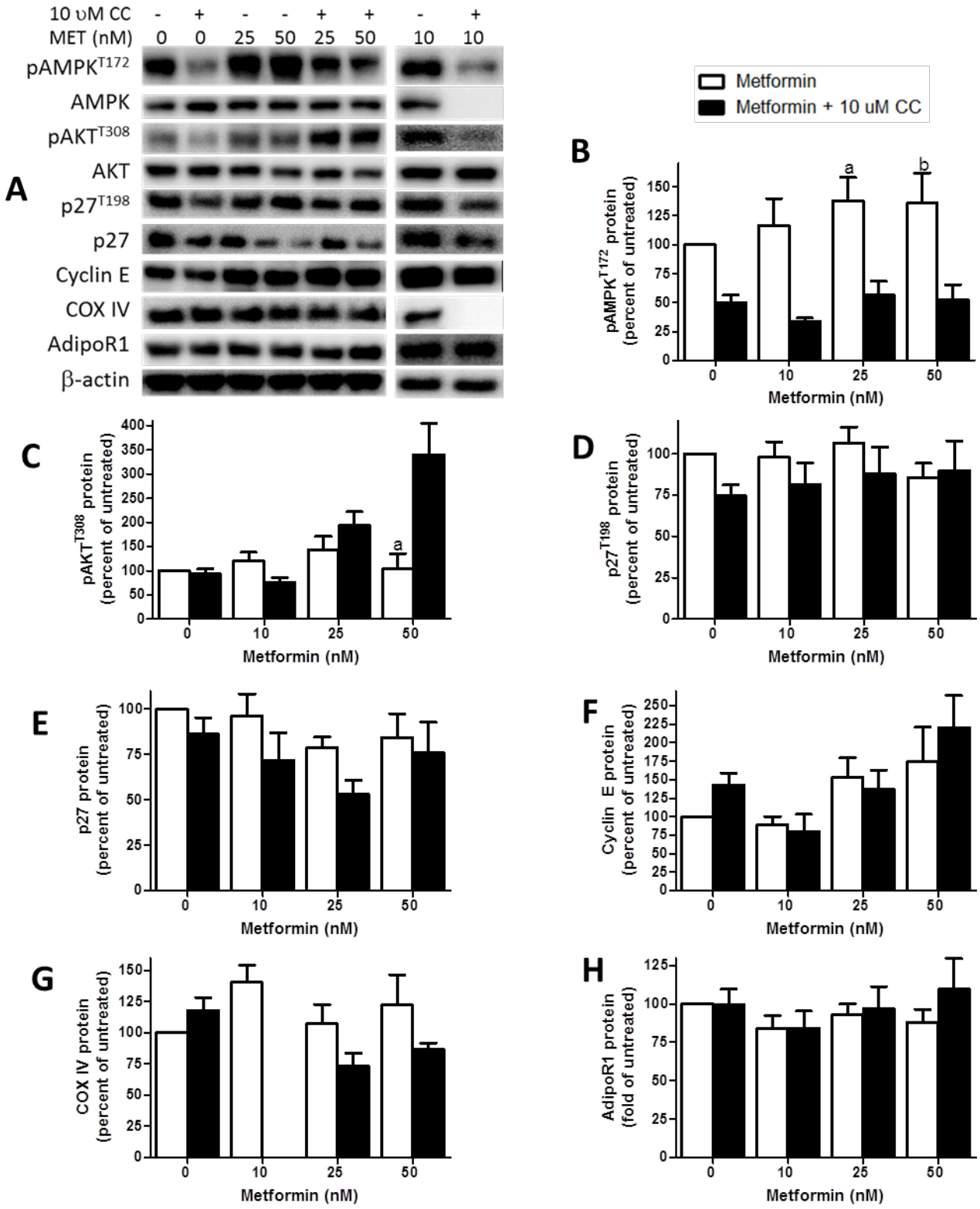

Figure 9 — PC3 cells treated with Metformin and Compound C.

(**A**) Representative blots for LNCaP cells for treated with MET and CC, (**B-H**) graphical representation of relative optical density versus control (Mean ± SEM). Blots standardized to β-actin to correct for loading. Graphs have been rearranged for clarity. Letters which differ represent a significant difference ($p<0.05$). n= 6-12/group

7.6 PC3 – Metformin, Leptin and Compound C Treatment

Since LEP is seen to be elevated in obesity, MET treatments were conducted in the presence of LEP to determine if MET can counteract the effects of LEP. CC treatment caused no effect on MET effects in LEP treated cells. $pAMPK^{T172}$, $pAKT^{T308}$, $p27^{T198}$, cyclin E, COX IV and AdipoR1 showed no significant differences (**Figure 10 A-D,F-G**). MET reduced p27 in the presence of LEP by nearly 2/3rds from 154 ± 31% to 58 ± 11% of levels observed in untreated control cells. In the presence of LEP and CC p27 levels decreased and this was not affected by MET treatment (**Figure 10 A,E)**.

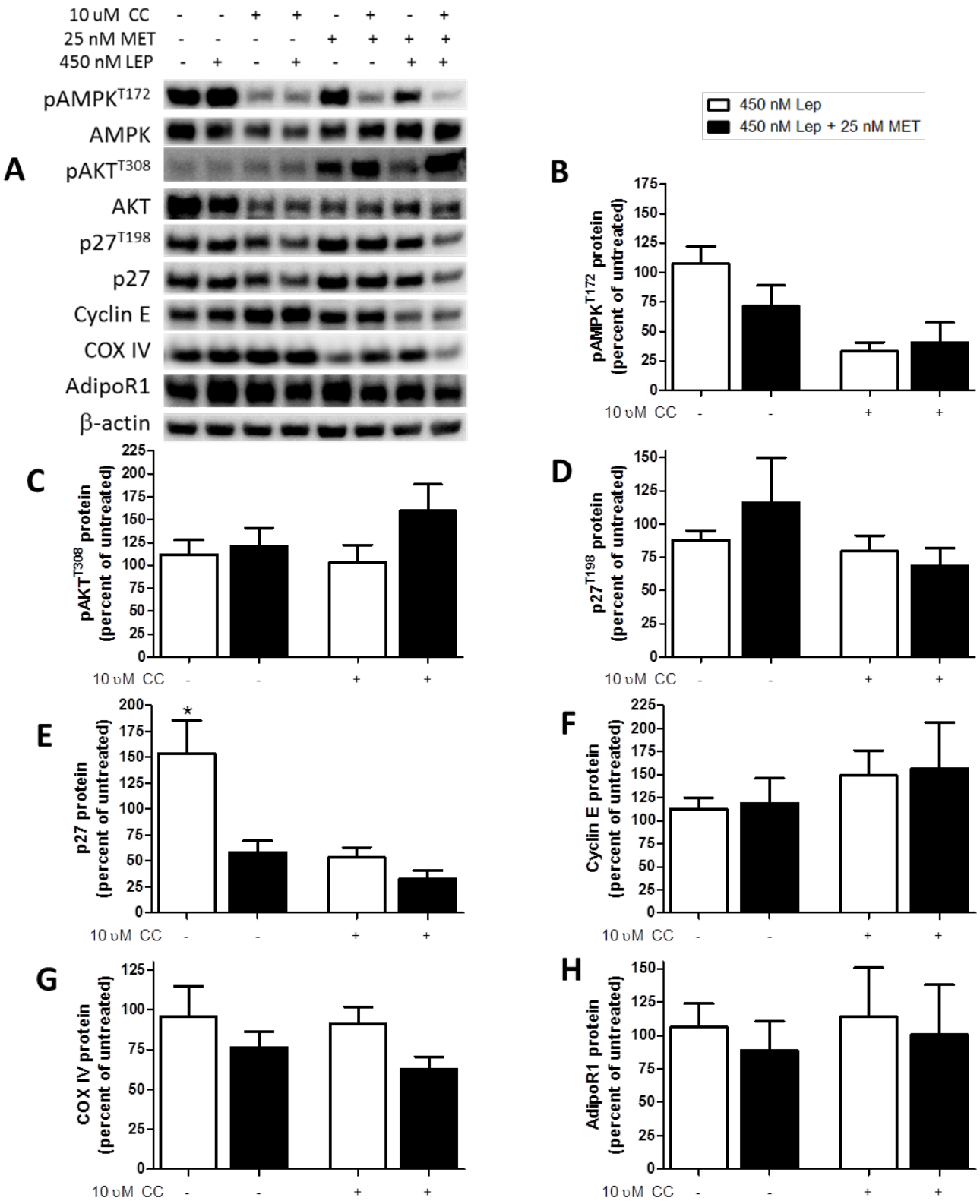

Figure 10 — PC3 cells treated with Metformin, Leptin and Compound C.

(**A**) Representational blots for LNCaP cells for treated with MET and CC, (**B-H**) graphical representation of relative optical density versus control (Mean ± SEM). Blots standardized to β-actin to correct for loading. Graphs have been rearranged for clarity. * represents significant differences between MET treated cells in the same CC same treatment ($p<0.05$). n= 6-12/group

7.7 PC3 – Conditioned Media Treatments

In order to determine the paracrine/endocrine effects of adipose tissue on PCa, PC3 cells were grown in the presence of CM prepared from human visceral adipose tissue. Addition of 25 nM MET to the CM caused a doubling of $pAMPK^{T172}$ protein levels with CM prepared from both abdominal adipose tissue (ACM) and periprostatic/perirenal adipose tissue (PCM), which is nearly a 60% increase from the effect of MET alone.CC in addition to the CM caused a decrease in $pAMPK^{T172}$ to their lowest point in each depot, with the lowest being 56 ± 5% for ACM. Similar to the results seen when MET was combined with CC, there was no effect of CC on $pAMPK^{T172}$ with MET in the presence of CM. The same pattern was present for the ACM and PCM. The 2 fold increase with MET treatment and 50% reduction of $pAMPK^{T172}$ with CC alone is more in line with our original hypothesis (**Figure 11 A,B**). $pAKT^{T308}$ protein levels in PC3 cells treated with ACM were decreased with the addition of MET, and a same result was observed in cells treated with PCM. When CC was added to PCM the addition of MET caused a reduction in $pAKT^{T308}$ protein levels that did not occour in the cells treated with ACM. The $pAMPK^{T172}$/$pAKT^{T308}$ antagonism that was hypothesized was more clearly visible in the presence of CM (**Figure 11 A,C**). Where MET caused an increase in $pAMPK^{T172}$ levels it caused a decrease in $pAKT^{T308}$ levels. CC surprisingly caused a reduction in both $pAKT^{T308}$ and $pAMPK^{T172}$ but a lesser effect on $pAKT^{T308}$.

p27 protein levels in PC3 cells treated with ACM was reduced with the addition of MET, which was not observed in cells treated with PCM. In both groups however the combination of CM and CC resulted in the highest levels of p27 at roughly 150% of untreated control cells (**Figure 11 A,E**). p27 ^{T198}, cyclin E, COX IV and AdipoR1 protein levels were unaltered by the CM treatments, similar to MET treatments alone (**Figure 11 A,D,F-H**).

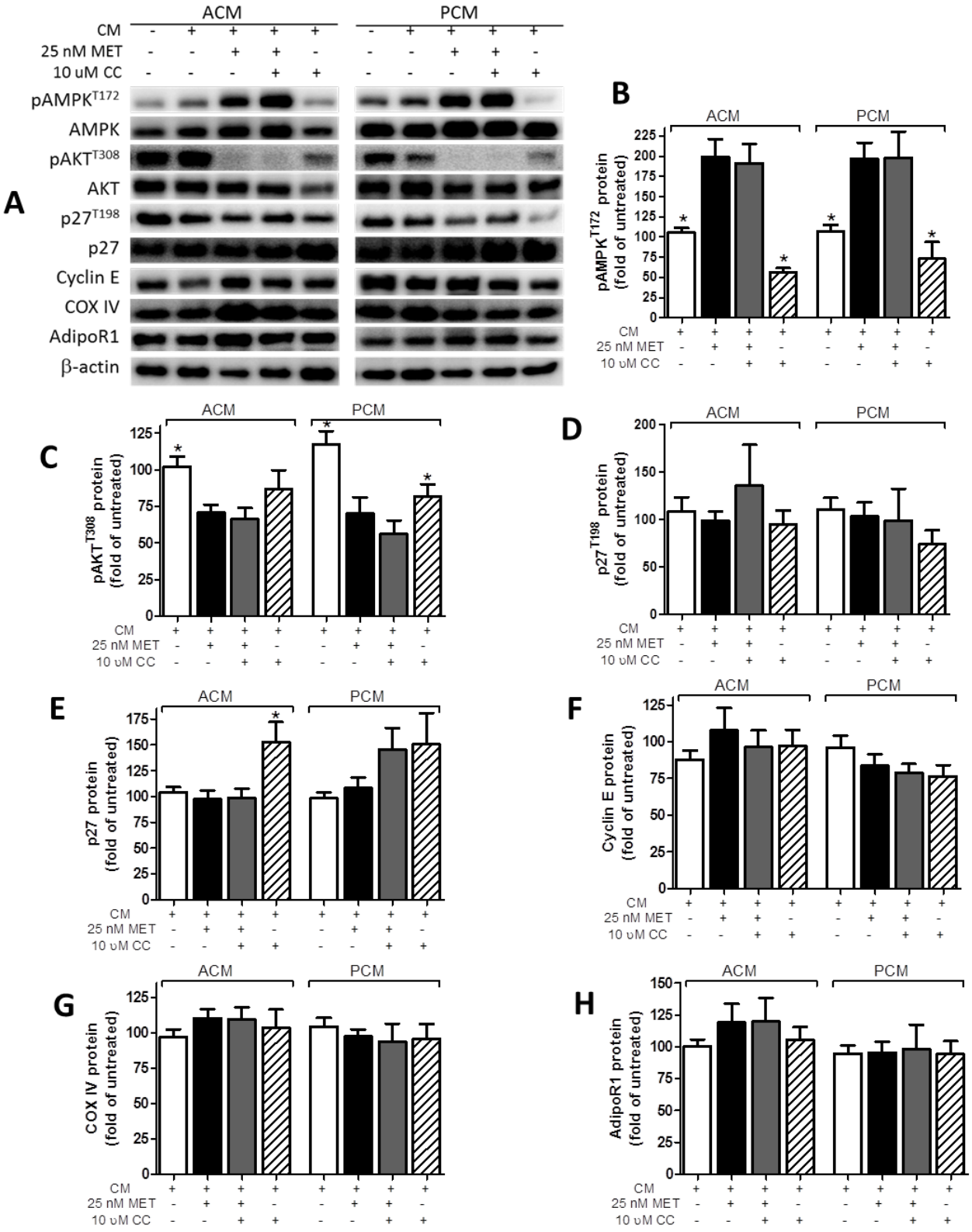

Figure 11 — PC3 cells treated with Metformin and Compound C in the Presence of Conditioned Media.
(**A**) Representational blots for LNCaP cells for treated with MET and CC, (**B-H**) graphical representation of relative optical density versus control (Mean ± SEM). Blots standardized to β-actin to correct for loading. Graphs have been rearranged for clarity. * represents significant differences between addition of MET treated cells ($p<0.05$). n= 16-35/group

7.8 PC3 – Conditioned Media Treatments Grouped by Cancer Type

In the existing results there were low numbers of patients/samples in the NCa and RCa group which makes proper statistical analysis difficult. PC3 cells treated with CM from adipose tissue, and grouped based on cancer type to see if there exists a difference in the paracrine effects of adipose tissue from patients with PCa, renal cancer (RCa) and patients without cancer (NCa).

$pAMPK^{T172}$ levels appear to be reduced in ACM for PCa and RCa patients when compared to NCa. A similar pattern was seen for total AMPK in ACM treated PC3 cells. For treatment with PCM a different pattern emerged, where PCa and RCa were higher than NCa. When corrected for the changing total AMPK levels the $pAMPK^{T172}$/AMPK ratio displayed a different pattern. For cells treated with ACM PCa was higher than RCa which was about the same as NCa, while for PCM PCa and RCa groups were about the same but lower than the NCa group (**Figure 12 A**). $pAKT^{T308}$ levels for cells treated with ACM were higher in the PCa group and lower in the RCa group compared to NCa patients. The same pattern was present in cells treated with PCM. Total AKT levels were observed to change thus a $pAKT^{T308}$/AKT ratio was utilized. The $pAKT^{T308}$/AKT ratio for cells treated with ACM was higher in the PCa group and lower in the RCa group compared to NCa patients, however for cells treated with PCM the PCa group was at the same level as the NCa group which were both higher than the RCa group (**Figure 12 B**). $p27^{T198}$ protein levels were unaltered in cells treated with ACM, however PCM caused an increase in $p27^{T198}$ protein levels in PCa when compared to NCa which was similar to the RCa (**Figure 12 C**). p27 protein levels like $p27^{T198}$ were unaltered with ACM treatment. With PCM treatment RCa had a higher value than PCa which in turn had a higher value than NCa (**Figure 12 D**). COX IV was unaltered by ACM, but with PCM PCa and RCa were similar and both higher than NCa (**Figure 12 F**). Cyclin E levels displayed a decreasing possible trend

for cells treated with ACM, with NCa being higher than PCa, which was higher than RCa, while cells treated with PCM displayed the opposite pattern (**Figure 12 E**). AdipoR1 protein levels in cells treated with ACM was elevated in PCa compared to NCa which was similar to RCa. In cells treated with PCM, AdipoR1 levels were highest in NCa group, lower in the PCa group and lowest in the RCa group, which might suggest a paracrine effect for AdipoR1, where it decreases with cancer from PCM versus ACM (**Figure 12 G**).

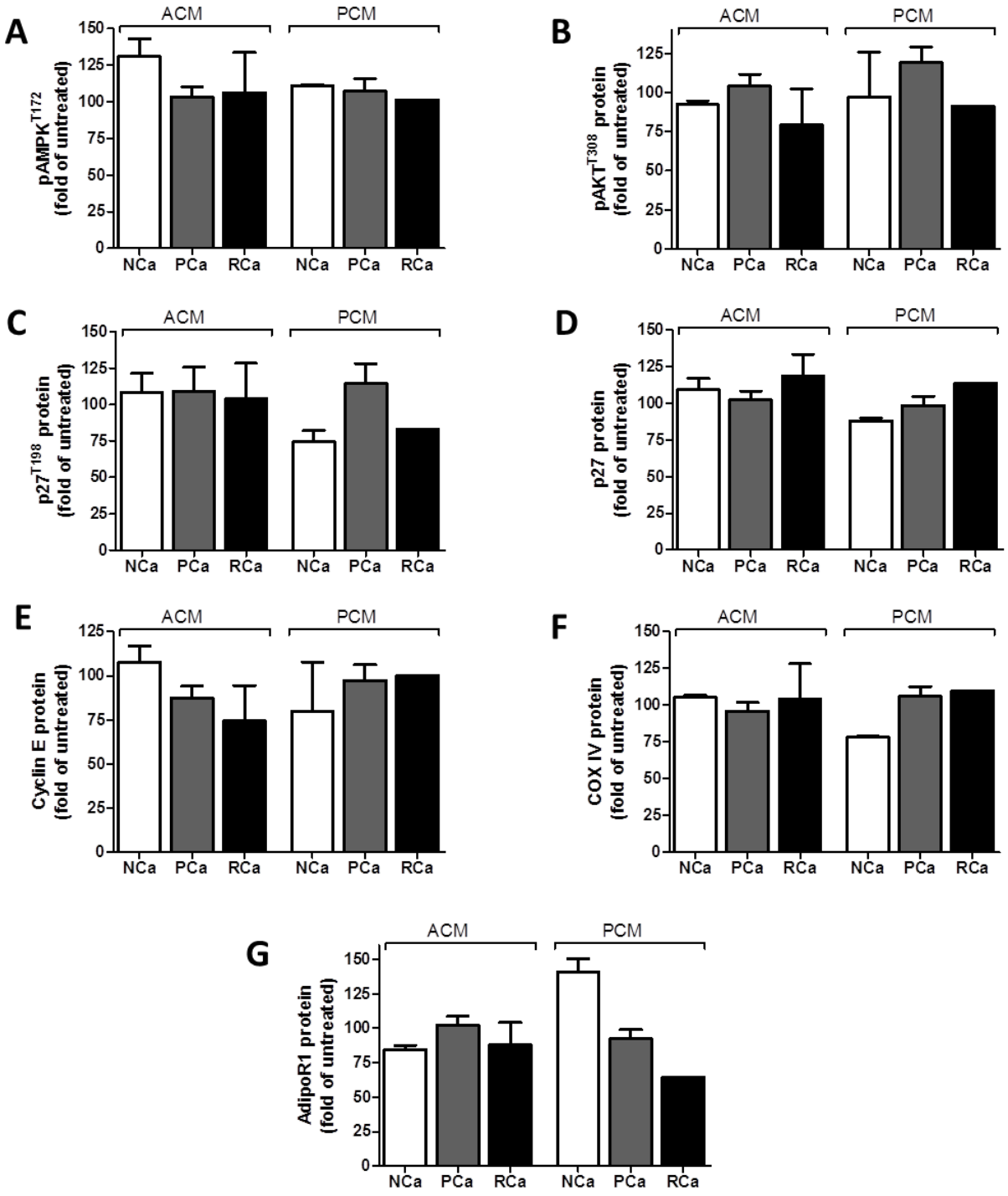

Figure 12 — PC3 cells treated conditioned media arranged by cancer type.
(**A-G**) graphical representation of relative optical density versus control (Mean ± SEM). Blots standardized to β-actin to correct for loading. Graphs have been rearranged for clarity. n= 1-31/group
NCa = non cancer, PCa = prostate cancer, RCa = renal cancer

8.0 Discussion:

The ever growing concern with obesity is a justified one. Prevalence of obesity in western countries is already high and continues to rise[1, 2]. It is not only seen in western countries but also in other countries around the world including Australia[7] and China[3], making it a global issue. One of the major comorbidities associated with obesity is a risk of developing certain types of cancer such as prostate cancer (PCa). Obesity does not only increase the risk of developing PCa[14], but is also associated with a worse prognosis in obese patients that develop PCa compared to healthy individuals of normal weight[106]. Obesity itself is also a risk factor for the development of T2D[13], which is also associated with a grimmer prognosis for patients who develop PCa[115]. However, research has shown that patients taking certain T2D medications (i.e metformin) are at a reduced risk of developing, and have a better prognosis if they do develop PCa[116, 122, 123, 125]. Thus, I tried to examine the cellular pathway in which MET acts as a protective factor against PCa in two different PCa cell lines.

Previous studies with breast cancer have shown that MET acts through the activation of AMPK resulting in cell cycle arrest[130]. MET clearly acts in an AMPK independent manner in androgen receptor positive LNCaP and androgen receptor negative PC3 cells. It was hypothesised that MET would activate AMPK, so experiments were conducted in the presence of Compound C (CC), which is an AMPK inhibitor, and thus would alter the results obtained using MET alone. In LNCaP cells CC did not affect pAMPK^{T172}, or AMPK total protein levels. However in PC3 cells CC was able to inhibit pAMPK^{T172} at all concentrations of MET to 50% of

the levels found in untreated control cells, and there was no concentration of MET that was able to overcome the effect of CC. CC did not alter other proteins of interest in both cell lines. The fact that other proteins of interest were not affected by CC clearly demonstrates that the mechanism of MET effects differentially involves AMPK in LNCaP and PC3 cells. Several studies have found that MET acts through an AMPK independent manner to reduce blood glucose levels in mice[136], as well as in human and rat glioma cells[137]. However MET has also shown to act via an AMPK dependent pathway in MCF7 breast cancer cells, to allow for cell cycle arrest effects on cyclin D1, and p27[130]. Also, MET has been found to have an AMPK dependant role on the inhibition of blocking lymphoma cell growth[138], suggesting that MET can act through an AMPK dependent or independent manner, depending of the type of cells.

Although LNCaP and PC3 cells were both discovered to be directly AMPK independent with regards to MET treatment, that's where the similarities ended. It is clear that in response to MET, LNCaP and PC3 cells behave differently. $pAKT^{T308}$ protein levels were increased with the combined treatment of CC and MET in PC3 cells. LNCaP cells on the other had had no changes with combination of MET and CC treatment, yet CC treatment alone increased p27 and $pAKT^{T308}$. Furthermore there were differences observed with MET treatment such as increasing $pAMPK^{T172}$ in PC3 cells, while $pAMPK^{T172}$ was decreased by MET in LNCaP cells. MET also caused decreases in $p27^{T198}$, cyclin E in LNCaP cells while $p27^{T198}$ was unaffected in PC3 and cyclin E trended towards increasing. Also $p27^{T198}$ protein levels displayed a similar pattern to that of $pAMPK^{T172}$ in LNCaP cells treated with MET. No such correlation was observed in PC3 cells, where $pAMPK^{T172}$ increased and no changes in $p27^{T198}$ were observed. Another difference was that both cell lines displayed a decrease in p27 protein levels, however at 100 nM MET PC3 cells increased to its highest value. All these differences clearly indicate that LNCaP and PC3

cells respond differently to treatment and this could potentially be due to the difference in presence of the androgen receptor, which can alter the intracellular machinery. It has been established that that activation of AMPK helps stabilize p27 by phosphorylating it at T198, and prevents its degradation[90, 91]. In LNCaP cells a similar pattern was observed for pAMPK^{T172} and p27^{T198}, yet p27 was not increased but rather appeared to decrease at higher concentrations. However this similar trend was not observed in PC3 cells. Instead an increase in pAMPK^{T172} was accompanied by no changes in p27^{T198} and a decrease in total p27, which would seem to suggest that in the more aggressive androgen receptor negative PC3 cells, the link between AMPK and p27 is dysfunctional or unimportant. This is in line with research that states that cancer cells that have reduced p27 or a lack of p27 regulation are more aggressive than cancer cells with p27 regulation[87, 139]. Since p27 was observed to increase at 100 nM of MET and it is known that p27 is protective with respects to cancer progression, we postulate that cells with low levels of p27 are susceptible to MET. However, at high concentration of MET the only cells able to withstand the toxicity are cells exhibiting high p27 levels, despite the low numbers.

With MET potentially causing a toxic environment at higher concentrations, we observed morphological changes and detachment from the plate in LNCaP cells. We postulate that in the less aggressive LNCaP cells treated with MET are undergoing autophagy or apoptosis. A recent study has found that there is an increase in pAMPK^{T172} levels and the ability to cells to enter a last ditch effort to survive through autophagy during external environmental stress[140]. It was determined that a certain threshold of AMPK activation is required for the cells to be able to utilize this desperate pathway to potentially survive external environmental stresses[140]. It has been shown that activation of AMPK by testosterone through androgen receptor supports proliferation and invasion in LNCaP cells[141, 142]. Our own results are consistent with an interation

between testosterone and AMPK in mediating intracellular signaling. This indicates that there is a link between testosterone and AMPK activation, and so the presence of the testosterone receptor impacts AMPK activation. Even though autophagy is seen as cell death, it can detrimental or crucial to cell survival. It has been found that autophagy at earlier stages of cancer progression is detrimental as allows the formation of free radicals as well as preventing new proteins from being formed and degrading already existing proteins, thus stopping progression[143]. While at later stages autophagy is induced as a protective mechanism to allow cancer cells, located in the central areas of the tumor to survive local low nutrient and low oxygen microenvironments[144, 145]. Initially we expected MET to increase the activation of AMPK in a dose dependent manner, which was not the case in LNCaP cells, where the addition of MET caused an initial increase followed by a decrease from 1 nM MET. Since MET caused an initial increase at 1 nM MET and then followed by a subsequent decrease at higher concentrations, it suggests that the environment could have become too "toxic" to allow the cells to survive. Since reduced AMPK activation was observed it seems that apoptosis may have been induced based on previous reports[140, 146]. Other studies have shown that in LNCaP cells CC has minimal effects of AMPK activation, similar to what was observed in our experiments[146]. Our results would suggest this with the decreased overall protein levels as well as the morphological changes and detachment that were observed, that at higher concentrations of MET the cells were undergoing apoptosis rather than autophagy. It has also been reported in other papers that activation of AMPK is required for cells to enter autophagy to utelize energy from destruction of intracellular components (autophagy) topotentially survive the external stresses imposed by the environment[147, 148].

To try to examine the role of MET in a more complete environment, CM was generated from two different visceral adipose sites for from PCa patients and subsequently used to treat PC3 cells, with MET, CC or a combination of both. The addition of MET to the CM caused an increase in AMPK activation from what was observed with MET treatment alone and was accompanied by a decrease in AKT activation in both depots. Thus, MET elicited opposite effects of AMPK and AKT. When MET was combined with CM it and CC it differed from what was observed in our previous experiment without the presence of CM. When PC3 cells were treated with MET and CC, CC reduced the levels of $pAMPK^{T172}$ to roughly 50% and no concentration of MET was able to override this inhibitory effect. When treated with CM, MET was able to override the inhibitory effects of CC on AMPK activation. MET was found to also have an additive effect in the presence of CM as it increased beyond what was seen in MET treatments alone. Since the addition of CC to MET in the CM did not cause a change it still agrees that MET acts via an AMPK independent manner. Thus MET treatment in CM is more in line with our hypothesis that MET activates AMPK which leads to AKT suppression. A possible explanation for this is that in the CM that is generated from adipose tissue, there are a plethora of other factors some of which are inhibitory while others being activators of cell cycle progression and that the responses of PC3 cells to MET in CM experiments may better represent what happens *in vivo*, as opposed to MET alone which appears to be causing an environment that is too toxic for the cells. This is consistent with other reports showing MET toxicity being specific to PCa cells[127].

Part of the initial study design was to examine if there are differential effects of CM generated from abdominal visceral adipose tissue (ACM) and CM generated from periprostatic/perirenal adipose tissue (PCM) on PC3 cell cycle regulation. Both depots elicited

similar results with a few exceptions suggesting that both visceral adipose depots contribute similarly to cancer growth. This suggests endocrine effects on growth microenvironment might dominate over paracrine effects. However, there were differential effects on the activation of AKT between the two different depots. $pAKT^{T308}$ had a 25% higher activation with PCM treatment versus ACM treatment. Also MET caused a greater decrease in $pAKT^{T308}$ protein levels in ACM. As with AMPK, CC had no effect on $pAKT^{T308}$ proteins levels with ACM treatment, but did cause a reduction with PCM treatment when combined with MET and CC. This suggests that there may be other adipose derived factors which affect which ones are expressed differentially by the 2 adipose depots.

As can be seen from this study, MET seems to be a promising area of research for PCa prevention and treatment. Overall MET seemed incredibly toxic to the cells on its own especially the LNCaP cells, and less so on the more aggressive PC3 cells. MET also lowers blood glucose levels in patients thus potentially lowering the amount of directly available fuel for cancer cells. It is uncertain if MET alters the metabolic environment of cancer cell, so that needs to be investigated but MET has direct effects on PCa cells that are AMPK independent, and has potential future in management and treatment of PCa in association with obesity.

9.0 Limitations and future directions:

As with any experiment or set of experiments there are always a certain set of limitations and improvements that can be made. One of our major limitations is that all experiments were done in cell cultures not in a whole body environment or *in vivo*. As we see from our experiments, treatment with MET alone produced different results than when MET was added to CM generated from patient adipose tissue. This approaches mimicking the tumor environment but it does not replicate it. For future studies these cells will need to be implanted into animals and try either injecting or ingesting MET to see if the metabolism of MET alters its action and to see how that action occurs *in vivo*.

With the results obtained we postulated that at higher concentrations of MET that the cells were undergoing some form of cell death, so markers of cell death would need to be utilized in the future to determine if MET is killing PCa cells, specifically the LNCaP cells. To determine if apoptosis is occurring markers such TUNEL assay and caspase cleavage could be utilized.To determine if autophagy is occurring Beclin I and LC3 II will need to be analyzed.

For our experiments, LNCaP and PC3 cells were used and tested. This allowed for comparisons between androgen receptor positive and androgen receptor negative cell lines. If the same experiments were to be conducted with DU145 cells which are also androgen receptor negative PCa cells, it will allow for a greater generalization between androgen receptor negative cells, in which we would expect similar results to the PC3 cells.

A limitation of this study is that number of patient samples that were obtained for use in CM treatment. The vast majority of cases were patients with PCa, and only a few that had NCa or RCa. This makes it more difficult to do analysis based on cancer type, but more patients of the

latter two groups are being obtained an analysed by our lab, to allow for a better comparison in the future. Also with regards to the patients, a lot of the patients were within the overweight group with a BMI of 25 to 29. More patients with BMI less than 25 and greater than 30 need to be recruited to the study to be able to observe is BMI has any effect on the cells prepared from their adipose tissue.

We are unsure if MET has the potential to alter the microenvironment or the properties of adipose tissue in patients who are on it for management of type II diabetes. A study can be designed with the same conditions as above to exam if patients on MET have a different adipokine profile compared to patients not on MET and to examine if this difference translates to different growth environments for PCa.

In order to conduct all the required experiments roughly 3 grams of adipose tissue is required from each depot. A drawback of the CM experiments was that we were limited by the amount of adipose tissue that was collected during surgery. We were limited by the amount of tissue obtained as sometimes only 0.2 grams of adipose tissue were obtained. Further guidelines and protocols need established with The Princess Margret Hospital of the amount of fat required for the experiments to try to obtain the required amount of adipose tissue to allow for all experiments. Also The Princess Margret Hospital might be limited in the amount of adipose tissue that they are able to be extract safely from each patient as some patients are learner than others.

To determine if MET as well as CM are arresting the cell cycle, FACS analysis needs to be conducted which is planned and will be done in our lab shortly.

Since all MET experiments in cell culture were conducted using LNCaP and PC3 cells for comparison, the LNCaPs should also be used for CM experiments, and it is currently being conducted in our lab. Alongside the LNCaP use for CM, experiments using the patients' serum, are being conducted in PC3 and LNCaP to determine if the patients serum has similar effects to those observed from CM generated from adipose tissue.

10.0 References

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11.0 Appendix A: Ethics

University Health Network
Research Ethics Board
10th Floor, Room 1056
700 University Ave
Toronto, Ontario, M5G 1Z5
Phone: (416) 581-7849

University Health Network
Toronto General Toronto Western Princess Margaret

Notification of REB Amendment Approval

Date: July 10th, 2012
To: Dr. Neil Fleshner
Rm. 130, 3rd Floor, 610 University Ave, Princess Margaret Hospital, Toronto
Ontario, Canada, M5G 2M9

Re: 10-0236-CE
The Effect of Cytokines Released from Periprostatic Fat on Prostate Cancer Growth

REB Review Type:	Expedited
REB Initial Approval Date:	May 5th, 2010
REB Amendment Approval Date:	July 10th, 2012
REB Expiry Date:	May 5th, 2013
Documents Approved:	
Amendment	Version date: June 20th, 2012
Consent Form	Version date: June 20th, 2012
Information Letter	Version date: June 20th, 2012
Documents Acknowledged:	
Protocol	Version date: June 20th, 2012

The UHN Research Ethics Board operates in compliance with the Tri-Council Policy Statement, ICH Good Clinical Practice Guidelines, Ontario Personal Health Information Protection Act (2004), Part 4 of the Natural Health Product Regulations and Part C, Division 5 of the Food and Drug Regulations of Health Canada.

Best wishes for the successful completion of your project.

Sincerely,

Larissa Potanina, MD
Research Ethics Coordinator

For: Anna Gagliardi, PhD
Co-Chair, University Health Network Research Ethics Board

There's always an answer. *We'll find it.*
Page 1 of 1

YORK
UNIVERSITÉ
UNIVERSITY

RENEWAL

Certificate #:	**2013 - 153**
Renewal Approved:	**05/12/14**
Approval Period:	**05/12/14/05/12/15**

OFFICE OF RESEARCH ETHICS (ORE)
5th Floor,
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4700 Keele St.
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Memo

To: Professor Micheal Connor, Faculty of Health, mconnor@yorku.ca

From: Alison M. Collins-Mrakas, Sr. Manager and Policy Advisor, Research Ethics
(on behalf of Duff Waring, Chair, Human Participants Review Committee)

Date: **Monday May 12th, 2014**

Re: Ethics Approval

The Effect of Adipokines released from Perioprostatic Fat Prostate Cancer Growth

---

With respect to your research project entitled, "The Effect of Adipokines released from Perioprostatic Fat Prostate Cancer Growth", the committee notes that, as there are no substantive changes to either the methodology employed or the risks to participants in the research project or any other aspect of the project, a renewal of approval of the above project is granted.

Should you have any questions, please feel free to contact me at: 416-736-5914 or via email at: acollins@yorku.ca.

Yours sincerely,

Alison M. Collins-Mrakas M.Sc., LLM
Sr. Manager and Policy Advisor,
Office of Research Ethics

RENEWAL

RESEARCH ETHICS: PROCEDURES to ENSURE ONGOING COMPLIANCE

Upon receipt of an ethics approval certificate, researchers are reminded that they are required to ensure that the following measures are undertaken so as to ensure on-going compliance with Senate and TCPS ethics guidelines:

1. **RENEWALS:** Research Ethics Approval certificates are subject to annual renewal.
    a. Researchers will be reminded by ORE, in advance of certificate expiry, that the certificate must be renewed
        i. Researchers have 2 weeks to comply to a reminder notice;
        ii. If researchers do not respond within 2 weeks, a final reminder will be forwarded. Researchers have one week to respond to the final notice;
    b. **Failure to renew an ethics approval certificate or** (to notify ORE that no further research involving human participants will be undertaken) **may result in suspension of research cost fund and access to research funds may be suspended/withheld** ;

2. **AMENDMENTS:** Amendments must be reviewed and approved **PRIOR** to undertaking/making the proposed amendments to an approved ethics protocol;

3. **END OF PROJECT**: ORE must be notified when a project is complete;

4. **ADVERSE EVENTS**: Adverse events must be reported to ORE as soon as possible;

5. **AUDIT:**
    a. More than minimal risk research may be subject to an audit as per TCPS guidelines;
    b. A spot sample of minimal risk research may be subject to an audit as per TCPS guidelines.

FORMS: As per the above, the following forms relating to on-going research ethics compliance are available on the Research website:

a. Renewal
b. Amendment

RENEWAL

c. End of Project
d. Adverse Event

YORK
UNIVERSITÉ
UNIVERSITY

Department of Occupational Health and Safety

MEMORANDUM

TO: Prof. Connor

FROM: Debbie Kolozsvari

SUBJ: Biosafety Training

DATE: October 18, 2011

Thank you for having your student attend the biosafety training session held in September 2011.

Please keep a record of training for all staff/students under your supervision that attend a session, which will be useful when completing your next Biosafety Certificate New/Renewal Application.

Attached is the training certificate card to be distributed to your staff/student.

Certificate of Training

Beshoy Nazeer

has successfully completed

BIOSAFETY TRAINING

YORK
UNIVERSITÉ
UNIVERSITY

Instructor: Debbie Kolozsvari, Biosafety Officer
Date: Sept. 13, 2011
Department of Occupational Health and Safety

University Health Network

CONSENT TO PARTICIPATE IN A RESEARCH STUDY

Title	The effect of cytokines released from periprostatic fat on prostate cancer growth
Investigator	Dr. Neil E. Fleshner (phone 416-946-4501 ext. 2899)

Introduction

You are being asked to take part in a research study. Please read this explanation about the study and its risks and benefits before you decide if you would like to take part. You should take as much time as you need to make your decision. You should ask the study doctor or study staff to explain anything that you do not understand and make sure that all of your questions have been answered before signing this consent form. Before you make your decision, feel free to talk about this study with anyone you wish. Participation in this study is voluntary.

Background and Purpose

We know that fat may promote prostate cancer growth, but it is not clear whether fat from beside the prostate plays a more important role in cancer development than fat from other areas of the body. By growing prostate cancer cells in the laboratory with different types of fat we should be able to learn if the site fat comes from plays any role in prostate cancer growth.

The goal of our study is to learn whether fat from along side the prostate or other parts of the body has an impact on prostate cancer growth.

You have been asked to take part in this research study because you, as a part of your standard care, are about to undergo abdominal surgery either for cancer or non-cancer reasons. During this surgery, it would be easy to remove a small piece of fat surrounding the organ you are having surgery on or from the surgical specimen for further experiments in a laboratory.

About 70 patients from the University Health Network hospitals (Toronto General Hospital and Princess Margaret Hospital) will be included in the study.

Study Visits and Procedures

During the already scheduled surgery, a surgeon from the research team will remove a small amount of fat (a thimble-full, <1cc) from the organ that is operated on as part of your surgery. Also, a similar amount of fat will be taken from just beneath the skin where your surgical incision was made. You will also have a blood sample drawn (1

tube, 5mL – 1/3 tablesoon) as part of the study. We look at proteins released by fat that exist in your blood; we will compare them to other patients' samples. In addition to these samples that will be taken, we will also measure your hip and waist sizes using a tape measure.

These procedures will not affect your diagnosis or treatment, will not harm you or cause any cosmetic defects, and will not increase your surgical time. The standard of care, which is the surgery you are undergoing, will not be compromised in any way. The samples removed will not produce any new information that could be medically useful to you as an individual.

Once you have had your surgery there will be no further follow-up visits related to the research study. However, you will continue your usual follow-up with your cancer specialist.

Risks Related to Being in the Study

There is very little risk to being in this study. We will require a blood sample and a small fat tissue sample from along side of your skin incision and from the organ you are having surgery on. This will all take place while you are asleep during your surgery. There will be no increase in the surgical time. If you are having an organ removed, the fat specimen will be taken from the organ after it is removed. The tiny amount of fat removed from the surgical incision will not cause any visual defects.

Despite there being minimal medical risks, being in this study may make you feel uncomfortable.

Benefits to Being in the Study

You will not receive direct benefit from being in this study. Information learned from this study may help other people with prostate cancer in the future.

Voluntary Participation

Your participation in this study is voluntary. You may decide not to be in this study, or to be in the study now and then change your mind later. You may leave the study at any time without affecting your care. You may decide to withdraw your samples from the research at any time. If you decide to do so, please let the study team know (Dr. Neil E. Fleshner or Karen Hersey) and your samples will be destroyed. However, any results already generated during the study will still be used for the study purposes.

We will give you new information that is learned during the study that might affect your decision to stay in the study.

Alternatives to Being in the Study

You do not have to join this study to receive treatment for your condition. This study does not affect treatment and type of treatment you receive for your condition.

Confidentiality

If you agree to join this study, the study doctor and his/her study team will look at your personal health information and collect only the information they need for the study.

Personal health information is any information that could be used to identify you and includes your: name, address, date of birth, new or existing medical records, that includes types, dates and results of medical tests or procedures.

The information that is collected for the study will be kept in a locked and secure area by the study doctor for 10 years. Only the study team or the people or groups listed below will be allowed to look at your records.

Representatives of the University Health Network Research Ethics Board may look at the study records and at your personal health information to check that the information collected for the study is correct and to make sure the study followed proper laws and guidelines.

All information collected during this study, including your personal health information, will be kept confidential and will not be shared with anyone outside the study unless required by law. You will not be named in any reports, publications, or presentations that may come from this study.

If you decide to leave the study, the information about you that was collected before you left the study will still be used. No new information will be collected without your permission.

In Case You Are Harmed in the Study

If you become ill, injured or harmed as a result of taking part in this study, you will receive care. The reasonable costs of such care will be covered for any injury, illness or harm that is directly a result of being in this study.

In no way does signing this consent form waive your legal rights nor does it relieve the investigators, sponsors or involved institutions from their legal and professional responsibilities. You do not give up any of your legal rights by signing this consent form.

Expenses Associated with Participating in the Study

You will not be paid or have to pay for any of the procedures involved with this study.

Conflict of Interest

There is no direct financial gain by any of the researchers involved in this study. All investigators involved have an interest in completing this study. Their interests should not influence your decision to participate in this study. You should not feel pressured to join this study.

Questions About the Study

If you have any questions, concerns or would like to speak to the study team for any reason, please call: Dr. Neil E. Fleshner at 416-946-4501 ext. 2899 or Karen Hersey at 416-946-4501 ext. 2155.

If you have any questions about your rights as a research participant or have concerns about this study, call the Chair of the University Health Network Research Ethics Board (REB) or the Research Ethics office number at 416-581-7849. The REB is a group of people who oversee the ethical conduct of research studies. These people are not part of the study team. Everything that you discuss will be kept confidential.

Consent

This study has been explained to me and any questions I had have been answered. I know that I may leave the study at any time. I agree to take part in this study.

Print Study Participant's Name	Signature	Date

(You will be given a signed copy of this consent form)

My signature means that I have explained the study to the participant named above. I have answered all questions..

Print Name of Person Obtaining Consent	Signature	Date

Was the participant assisted during the consent process? ☐ **YES** ☐ **NO**
If **YES**, please check the relevant box and complete the signature space below:

☐ The person signing below acted as a translator for the participant during the consent process and attests that the study as set out in this form was accurately translated and has had any questions answered.

Print Name of Translator	Signature	Date

Relationship to Participant	Language

☐ The consent form was read to the participant. The person signing below attests that the study as set out in this form was accurately explained to, and has had any questions answered.

Print Name of Witness	Signature	Date

Relationship to Participant